The genetics of adaptive photoperiodic response in Nasonia vitripennis

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The genetics of adaptive photoperiodic response in *Nasonia vitripennis*

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This research has been carried out at the Evolutionary Genetics group, which is part of the Centre for Ecological and Evolutionary Studies (CEES) of the University of Groningen (The Netherlands), according to the requirements of the Graduate School of Science (Faculty of Mathematics and Natural Sciences, University of Groningen)

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General introduction[1]

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[1] Excerpts of this chapter have been published in the review paper: Hut, R.A., Paolucci, S., Dor, R., Kyriacou, C.P. & Daan, S. 2013. Latitudinal clines: an evolutionary view on biological rhythms. *Proc. R. Soc. B Biol. Sci.* 280: :20130433. The PhD candidate is a co-author

INTRODUCTION

The term “adaptation” refers to the process through which populations become better suited to their present environment. A particular phenotype that confers a reproductive advantage in a given environment is selected by natural selection and the genetic (heritable) basis of that phenotype becomes more common in the population. This process takes place over many generations and may eventually lead to adaptive evolution, emergence of novel traits and speciation resulting in a proper match between the organism and the environment in which it lives (Orr, 2005). Adaptive traits are phenotypic traits (often determined by many genes) that allow an efficient use of natural resources in such a way that reproductive success is maximized. Within and among populations these traits show large phenotypic variation as result of adaptation to specific environments. This variation is often based on the contribution of different factors: genetic differences, environmental effects, phenotypic plasticity and interaction between genetic and environmental effects. Understanding the relative contribution of each factor in shaping phenotypic adaptive traits remains one of the key goals of evolutionary biological research.

Recently, the advent of modern genomic techniques allowed a rapid increase of the knowledge on species adaptation and contributed to the investigation of fundamental questions on the genetic basis of adaptive evolution. Among others: does adaptation arise from standing genetic variation or does it involve new mutations? Are there many genes responsible for adaptation or few genes with pleiotropic phenotypic effects? What is the role of phenotypic plasticity in evolution?

The work presented in this thesis represents a contribution to the understanding of adaptation genetics as it investigates the genetic basis of seasonal photoperiodic diapause, an adaptive trait that allows organisms to synchronize their life cycle with seasonal environmental cycle.

Seasonal adaptation

Adaptation of organisms to seasonally changing environments involves the ability to cope with cyclic and persistent environmental changes. Because most biotic and abiotic sources that organisms can utilize are directly or indirectly related to seasonal changes, individuals must be able to synchronize their life cycle with annual cycles of environmental factors in order to maximize the exploitation of favourable seasons and minimize the exposure to unfavourable seasons. Organisms respond to seasonal environmental changes through a wide range of adaptations that involve physiological, behavioural and developmental modifications.

Almost all organisms, ranging from bacteria and fungi to plants, invertebrates and vertebrates, have evolved some kind of ability to cope with seasonal changes. Insects are the most diverse group of animals on the planet, they are widely spread throughout the world and their seasonal adaptation strategies evolved under the pressure of many different seasonal environments. Therefore, insects represent a highly interesting group to study the evolution and variation of seasonal adaptations.

Diapause

Diapause is a physiological state of dormancy mediated by hormones, used by several insect species (and other invertebrates) to survive adverse environmental conditions occurring on a seasonal base, such as cold winter or dry and hot summer. Because of its crucial role in seasonal regulation of life cycle, diapause is considered an important adaptive life history trait, shaped by natural selection.

Diapause is induced and regulated by environmental cues that are called *token stimuli* (Tauber *et al.,* 1986). These environmental stimuli do not constitute, in themselves, favourable or unfavourable conditions for growth, development and reproduction but they signal an upcoming change in environmental conditions (for example approaching winter). In this regard, one of the most important aspects of diapause is its anticipatory nature: insects are able to perceive environmental cues long in advance of the diapausing stage itself, the information is stored for a number of developmental stages and sometimes even generations and then translated into morphological, physiological and behavioural modifications characteristic of diapause induction. Thanks to its predictive value, diapause allows harmonization of the entire life cycle with seasonal cycle of the environment. This latter feature is essential to distinguish diapause from other types of dormancies, such as quiescence, which allow prompt response to sudden, unpredictable short-term environmental changes and are thus considered consequential responses.

In an insect's life history, diapause typically occurs at a specific stage and during a specific season. Closely related species can differ in their diapausing stages and for each species the diapause responsive

stage(s) is genetically determined. The developmental stage in which token stimuli are perceived (sensitive stage) and the diapause stage do not always overlap, they might be widely separated within the same generation or even between generations (Fig. 1.1). Species in which the sensitive stage occurs in the female parent and the response in the offspring are often referred to as having a "maternal effect" diapause. Examples of maternal effects in diapause response are reported for parasitic Hymenoptera, sarcophagid flies, mosquitoes and crickets (reviewed in Tauber *et al.*, 1986 and Mousseau & Dingle, 1991). In other species, especially those that undergo diapause as adults, the sensitive stage and the responsive stage overlap (e.g. *Drosophila*).

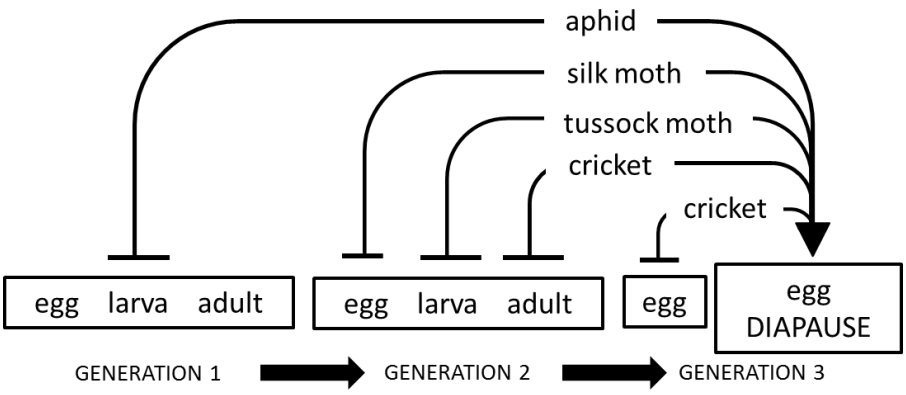

Figure 1.1 Example of insect species that undergo diapause in the egg stage and differ in the stage that perceives diapause-inducing stimuli. Sensitive stage and diapause stage are often separated within or between generations. (modified from Tauber *et al.,* 1986)

Token stimuli: environmental factors inducing diapause

An insect's set of physiological diapause response is genetically determined but environmental factors determine whether or not and to what degree diapause will occur (Tauber *et al.*, 1986). Photoperiod (length of daily light) is considered the most important factor involved in diapause induction. Nearly all physical factors display considerable variation in their seasonal change both in space (different localities) and time (different years). However, photoperiod is not affected by changes from year to year and is fixed for a given place on Earth. Its predictability and persistency provide the most reliable cues to future conditions, especially in temperate zones where changes in day length are highly correlated with changes in temperature and food availability. Usually the photoperiodic response of insects is represented by the percentage of individuals entering diapause as a function of stationary photoperiods. The result is a typical photoperiodic response curve (Fig. 1.2) that rises very steeply as it passes through the *critical photoperiod* which corresponds to the photoperiod that elicits 50% response. The steepness of the photoperiodic response curve reflects rigorous natural selection for the timing of diapause induction (Tauber *et al*., 1986 and references therein).

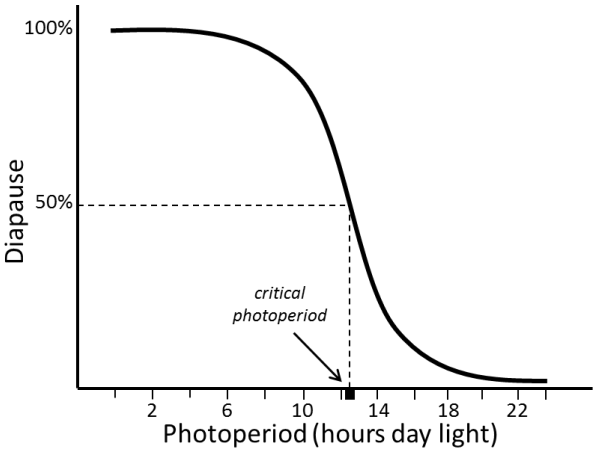

Figure 1.2 Typical photoperiodic response curve of a long-day insect showing diapause induction at short photoperiods and diapause inhibition at long photoperiods. The photoperiod that elicits 50% of the diapause response is called *critical photoperiod*.

Next to photoperiod, the most important factor affecting diapause is temperature. Although unpredictable, temperature has a regular seasonal pattern and insects evolved various responses to seasonal changes in temperature. In species with winter diapause, low temperatures induce diapause, while summer diapause is induced by high temperatures. In some temperate species temperature represents the major environmental cue inducing diapause. In most insect species, temperature interacts with photoperiod in several ways to induce diapause (Danilevskii, 1965; Beach, 1978; Gomi, 1997; McWatters & Saunders, 1998; Saunders, 2002; Ahmed *et al.*, 2007). In general the thermal modification of photoperiodic response is expressed as alteration of critical photoperiod: low temperatures increase the critical photoperiod and high temperatures decrease it (Fig. 1.3).

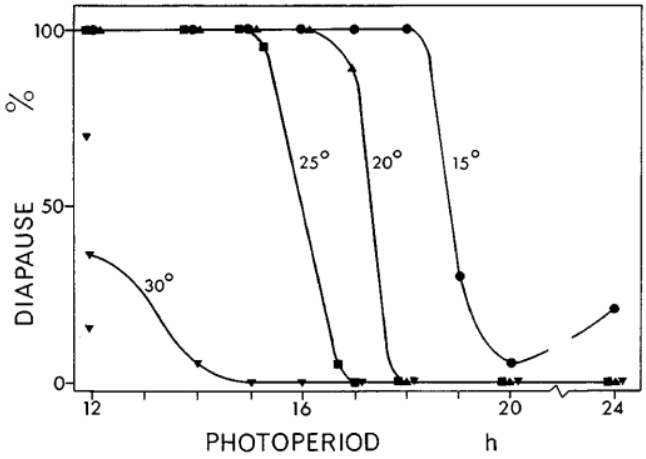

Figure 1.3 Modulating effect of temperature on critical photoperiod inducing diapause in the moth *Acronycta rumicis.* The critical photoperiod is shorter at high temperatures and longer at lower temperatures (Danilevskii, 1965).

Although photoperiod and temperature serve as primary stimuli regulating the seasonal cycles of most insect species, other environmental factors can be involved. For example, food availability and humidity represent important resources that vary seasonally and insects are able to time their life cycle with these factors. In summary, under natural conditions, insects are exposed to different environmental factors that interact with each other and, under certain circumstances, induce diapause. In order to determine the influence and the relative importance of single factors, appropriate experiments should be designed in which environmental (experimental) conditions are carefully controlled and different factors are considered separately.

Variation in seasonal response

In temperate zones, nearly all environmental physical factors show seasonal changes (light, temperature, humidity, etc.) with a considerable annual variation. Species with wide distribution encounter a great diversity of climatic conditions and variability in seasonal conditions among localities. This is reflected in correspondingly large variation in life cycles as a result of local adaptation.

Inter-population variation in diapause response is usually associated with geography and especially with latitude. As climatic conditions vary from place to place along latitudinal gradients, almost any phase of diapause and other types of seasonal responses show latitudinal clinal variation. In particular, annual photoperiodic changes are strictly linked to latitude leading to latitude-specific selection pressure along North-South gradients (Fig. 1.4). This clinal variation has generated corresponding latitudinal clines in photoperiodic responses that have been observed in several organisms. In the next paragraph I will review different aspects of photoperiodism that vary according to latitude with special focus on insect diapause.

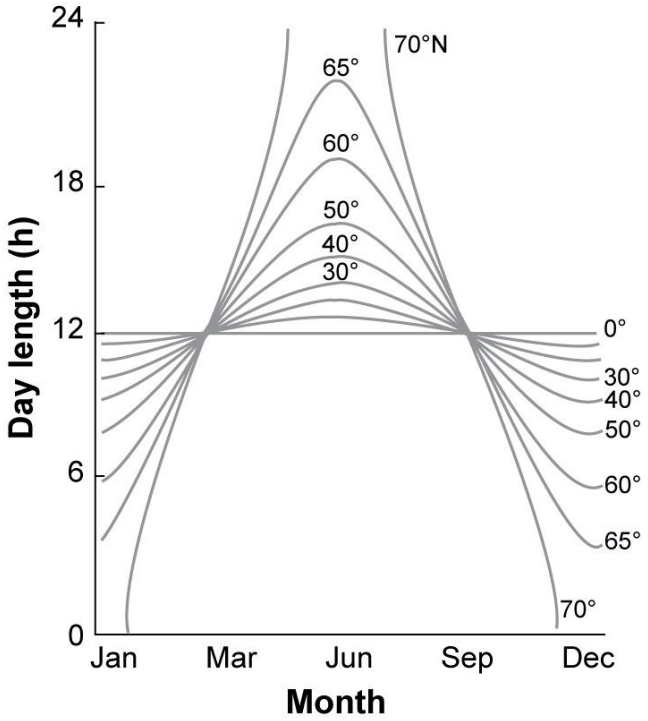

Figure 1.4 Seasonal patterns in day length (sunrise to sunset) at different latitudes (°N) in the northern hemisphere (Bradshaw & Holzapfel, 2007)

Latitudinal clines in diapause

Latitudinal variation in photoperiodism has been investigated mainly in insects in which short photoperiods trigger the induction of diapause, a form of winter dormancy. Danilevskii (1965) was the first to demonstrate a positive correlation between latitude and critical photoperiod for diapause induction. He showed that critical photoperiod increases by about 1 h every 5–6° latitude in the knot grass moth *Acronicta rumicis* (Fig. 1.5A). Positive correlations between critical photoperiod and latitude were later described in many other insects with various diapause stages, including

the adult reproductive diapause in the Japanese flower bug *Orius* (Shimizu & Kawasaki, 2001) and in the Finnish malt flies *Drosophila montana* (Tyukmaeva *et al.*, 2011), the pupal diapause in the butterfly *Sericinus montelus* (Wang *et al.*, 2011) and the maternally induced larval diapause in the parasitoid wasp *Nasonia vitripennis* (Paolucci *et al.*, 2013; chapter 2 of this thesis). The combined data reveal a general positive correlation between critical photoperiod and latitude, with an increased slope above approximately 40° N (Fig. 1.5B)

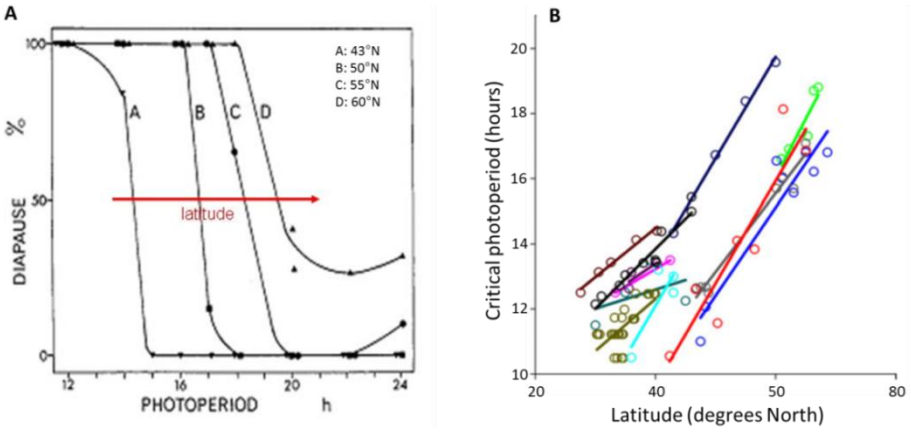

Figure 1.5 Latitudinal cline in photoperiodic diapause response. **A)** First study on latitudinal cline in photoperiodism, showing the effect of latitude on photoperiodic response curve (measured at 23°C in larvae of *Acronycta rumicis* (Danilevskii, 1965). **B)** Latitudinal clines in critical photoperiod for diapause induction in different insect species (Hut *et al.*, 2013 and references therein): Brown, *Sericinus montelus* (pupae); black, *Wyeomyia smithii* (larvae) ; purple, *Bruchidius dorsalis* (larvae); pink, *Chrysopa carnea* (adult); turquoise, *Homoeosoma electellum* (larvae); khaki, *Tetranychus puearicola* (adult); cyan, *Orius sauteri* (adult); dark blue, *Acronicta rumicis* (larvae); red, *Nasonia vitripennis* (larvae; maternally induced); green, *Drosophila montana* (adult); grey, *D. phalerata* (adult); blue, *D. transversa* (adult)

The latitudinal cline in critical photoperiod for diapause induction has adaptive significance related to the seasonal cycles at different latitudes:

in northern latitudes, the onset of winter occurs early in the year when days are still long, thus a long critical photoperiod allows proper diapause timing, whereas the short critical photoperiod of southern populations permits to enter diapause later in the year in order to fully exploit the longer favourable season (Fig. 1.4). Selection pressure on timing for photoperiodic diapause induction is also evident in studies on species that have recently entered new environments. Alien insects can adapt to the seasonal cycles of a newly colonized geographical range by the rapid establishment of latitudinal clines in critical photoperiod (Gomi, 2007; Sadakiyo & Ishihara, 2011; Bean *et al.*, 2012). Similarly, rapid change in critical photoperiod also occurs in response to global warming, as documented in northern populations of the pitcher plant mosquito *Wyeomyia smithii*, which shifted its photoperiodic response towards shorter photoperiods (Bradshaw & Holzapfel, 2001).

The power of photoperiod as indicator of seasonal change diminishes towards lower latitudes where different cues can interact with day length and modify the photoperiodic response. Temperature plays an important role as modulating factor in southern populations of the mosquito *Aedes atropalpus* (Beach, 1978) and in the fly *Calliphora vicina* (McWatters & Saunders, 1998), where higher temperatures suppress diapause incidence and shorten the critical photoperiod. The larger temperature sensitivity of southern populations allows more flexibility of the photoperiodic response in locations where occasional warm conditions in late summer permit an extension of the reproductive season before winter. Moreover, Saunders showed that thermoperiod (daily temperature cycles) alone can induce diapause in *N. vitripennis*

(Saunders, 1973), but it is not known whether this thermoperiodic response varies across latitudinal gradients.

Another aspect of diapause showing latitudinal variation is the incidence of diapause expressed as proportion of individuals that undergo diapause in a given population. In some insect species the number of generations per year is strictly constant throughout their geographic distribution. These species are usually univoltine and undergo an obligatory diapause regardless of environmental conditions (Sokolova, 2007). Many other species with wide latitudinal distributions express variation in the number of generations per year (bivoltine, multivoltine or non-diapause life cycle); typically southern populations have a higher number of generations in a year. This variation in voltinism is accomplished by the variable incidence of diapause at different locations. In most species the proportion of individuals entering diapause is higher in populations inhabiting northern latitudes. Diapause phenotype is favoured at higher latitudes where scarce natural resources due to short summers permit only one or two generations per year. In contrast, southern latitudes will favour non-diapausing individuals and will enable many generations per year.

Genetic control of photoperiodic response: role of circadian clock genes

The two major rhythms of the biosphere are the daily cycle evoked by rotation of the Earth around its own axis and the annual cycle derived from the elliptical orbit of the Earth around the sun. The consequences of these two celestial movements for the environment are the daily environmental light:dark cycles and the seasonal cycles which have deeply influenced the evolution of circadian and circannual rhythmicity of

organisms. The selection pressure from the photoperiodic daily cycle led to the evolution of internal circadian clock mechanisms that most species use to regulate their cyclic biological processes and to harmonize their life cycle with external environment. In fact, circadian clock machineries are entrained by environmental light:dark cycles and control daily timing of many molecular, cellular, physiological and behavioural activities.

Because of their strict link with photoperiodic cycles, circadian clocks are believed to be involved in seasonal photoperiodic responses. The functional involvement of the circadian clock system in photoperiodism and the evolutionary link between the two clocks has been proposed more than 70 years ago (Bünning, 1936). Subsequent experiments in various species have supported this hypothesis (reviewed in Goto, 2013). However, the study of the mechanistic details of the relationship between the two types of clocks is limited by the current knowledge on the molecular and genetic basis of the seasonal photoperiodic clock. This is still scarce compared to the well characterized molecular basis of circadian rhythmicity in model species such as *Drosophila melanogaster*. Research in this field has focused on the investigation of the role of circadian clock genes on seasonal photoperiodic responses. These genes are responsible for the internal daily rhythmicity and represent candidates for the genetic basis of diapause and other photoperiodic responses.

One approach to investigate the mechanistic details and evolution of the circadian clock and seasonal photoperiodic response is the study of clock gene polymorphism in natural populations and their association with different diapause responses. Natural genetic variation at two important

clock genes (*period* and *timeless*) has been investigated in detail in *D. melanogaster*. The gene *period* is present in natural populations in two main forms (alleles) which differ in the length of a stretch of DNA encoding for alternating threonine-glycine (Thr-Gly) amino acids. In European populations, the frequencies of the two alleles varies according to latitude, in particular $(Thr\text{-}Gly)_{20}$ is present at higher frequency in northern populations, opposite to $(Thr\text{-}Gly)_{17}$ which is more frequent at lower latitudes. A similar, albeit weaker, cline was observed in Australia (Fig. 1.6A) (reviewed by Kyriacou *et al.*, 2008). The second well known latitudinal study in clock gene polymorphism in *D. melanogaster* involved the gene *timeless*, another crucial gene in the molecular clock mechanism of the fruit fly. This gene is also present in European populations in two variants: *s-tim* and *ls-tim.* The first variant encodes for one single isoform of the TIM protein and the second type leads to the production of two isoforms due to a single nucleotide insertion allowing for two initiation codons and thus two transcripts that differ in length. The *ls-form* is present at higher frequency in populations from southern latitudes (Fig. 1.6B). Flies carrying different *tim* variants show differences in diapause tendency indicating a possible role of the *tim* gene in diapause (Tauber *et al.*, 2007). Surprisingly the *ls-tim* flies (present at higher frequency in the South) show a higher incidence of diapause that contrasts with the expectation of geographic distribution in diapause incidence. Subsequent phylogenetic and spatial analysis revealed a recent origin of the *ls-tim* allele and a recent diffusion in Europe, as a consequence of the photoperiodic environment which is absent in sub-saharian regions, the native area of *Drosophila melanogaster*. The conclusion was that

directional, not balancing selection, is acting on the *ls-tim* allele (Tauber *et al.*, 2007). Although these studies on latitudinal clines in gene polymorphism provided initial evidence for a possible evolutionary link between circadian clock and adaptive seasonal photoperiodic response, it remains unclear how the circadian clock governs the photoperiodic response and whether the clock genes play their role as single genes or as part of a circadian module (Emerson *et al.*, 2009).

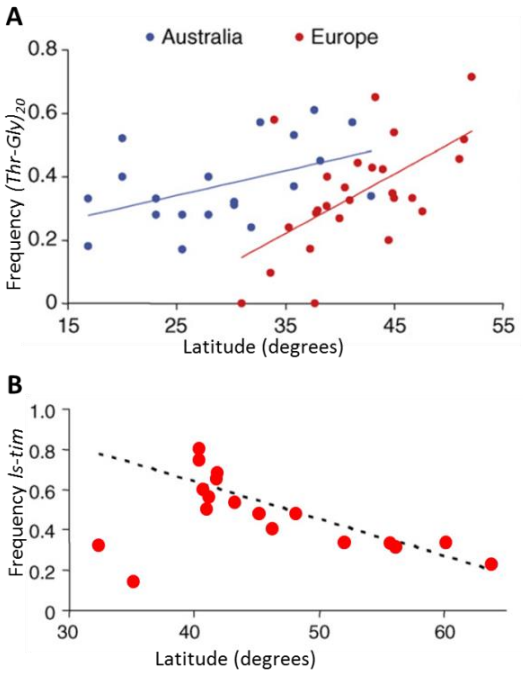

Figure 1.6 Latitudinal cline in clock gene polymorphism. **A)** Latitudinal cline in *period* polymorphism in Europe and Australia **B)** Latitudinal cline in *tim* polymorphism in Europe (Kyriacou *et al.*, 2008)

In recent years, research on the involvement of clock genes in photoperiodic responses has benefitted from the development of techniques such as RNA interference, use of mutants or expression analysis (reviewed in Goto, 2013). Studies performed in the pitcher plant

mosquito *W. smithii* (Mathias *et al.*, 2007), *Chymomyza costata* (Stehlík *et al.*, 2008), *Drosophila triauraria* (Yamada & Yamamoto, 2011), the flesh fly *Sarcophaga bullata* (Han & Denlinger, 2009) and the bean bug *Riptortus pedestris* (Ikeno *et al.*, 2011) revealed that clock genes are somehow involved in photoperiodic diapause. However, it is not known whether naturally occurring variants of clock genes have specific function in shaping the photoperiodic response in different environments. Moreover, it is unclear to what extent their potential effect is an integrated part of the circadian clock or whether it is just due to pleiotropic gene effects. The debate on the evolutionary link between the circadian clock and photoperiodism remains still open.

Despite the interest in clock genes as key candidates for studying the genetic basis of diapause variation in insects, other non-clock genes have been investigated for their potential role in natural diapause variation. For example the gene *Dp110* coding for the insulin regulated phosphatidylinositol 3-kinase (PI3-kinase) is part of the insulin signalling pathway that regulates reproductive diapause in *D. melanogaster*. Allelic variation in this gene is associated with latitudinal increase in the incidence of diapause in northern locations in North America. The gene is also important for its pleiotropic effects on other fitness-related traits (life span, fecundity, stress resistance) that vary with latitude and are genetically correlated to diapause (Williams *et al.*, 2006). Similarly, molecular variation in the gene *couch potato* in *D. melanogaster* leads to an amino acid polymorphism which shows clinal variation along the East coast of North America and correlates with variation in diapause incidence (Schmidt *et al.*, 2008). Natural variation in the same gene has been found

in Australian *D. melanogaster* populations, but it was not directly associated with diapause variation (Lee *et al.*, 2011).

Clearly, photoperiodic diapause induction is a complex trait which involves a cascade of physiological changes and differential expression of multiple genes. It is likely that several genes play different roles in the adaptive variation of diapause with small and large effects. The extent to which these genetic architectures differ between insect groups remains a fascinating question.

THE *NASONIA* MODEL SYSTEM

Haplodiploid wasps of the genus *Nasonia* have recently become a model system for research in various fields of evolutionary biology, including genetics of adaptation and speciation, ecology, development and behaviour. Population genetic studies and research into adaptive strategies are facilitated by the presence of four closely related species that vary in many aspects of their biology and in their geographic distributions: *Nasonia vitripennis* is widespread throughout the world, in the western part of North America it occurs in sympatry with *Nasonia girault*i and *Nasonia oneida* and in the eastern part with *Nasonia longicornis*. In nature, the four species are reproductively isolated due to infection with species-specific strains of the *Wolbachia* bacteria that cause cytoplasmic incompatibilities and hybrid breakdown (Breeuwer & Werren, 1990), but interspecific crosses are possible in the laboratory after antibiotic treatment and produce viable hybrid offspring.

One of the most important features of *Nasonia* wasps is the haplodiploid sex determination system, i.e. males are haploid and develop from unfertilized eggs, females are diploid and develop from fertilized eggs. The haploid genetics in males facilitates genotyping, haplotype study and evaluation of gene interactions (Pultz & Leaf, 2003; Werren *et al*., 2010). Recently, the complete genome of *Nasonia* has been sequenced and several molecular and genetic markers are now available (Werren *et al.*, 2010). Other properties that make *Nasonia* an excellent model organism include the short generation time, large family size and easy laboratory maintenance.

Nasonia *life cycle*

Nasonia is a gregarious parasitic wasp which uses the pupae of various fly species as hosts. The flies that are parasitized by *Nasonia* are mostly found in bird nests or carcasses. When a foundress adult female finds a suitable host, she drills through the host puparial wall with her ovipositor, injects venom which kills the fly, and lays eggs that develop inside the host. The wasp larvae feed on the host, pupate and emerge as adults from the host puparium. The entire development time varies depending on the temperature; it is two weeks at 25°C and three weeks at 20°C under laboratory conditions. In *Nasonia vitripennis*, males typically emerge 1 or 2 days before females, they stay on the host and wait for the emergence of the females. When both sexes have emerged, males perform courtship behaviour and subsequent mating usually occurs on the natal place or in the vicinity. After mating, the females disperse in search for new host patches where the cycle starts again, whereas males remain in the vicinity

of the host and die after few days. A scheme of the *Nasonia* life cycle is shown in Fig. 1.7. In temperate zones, *Nasonia* wasps overwinter as diapausing larvae inside the host puparium and resume development in spring when conditions are favourable.

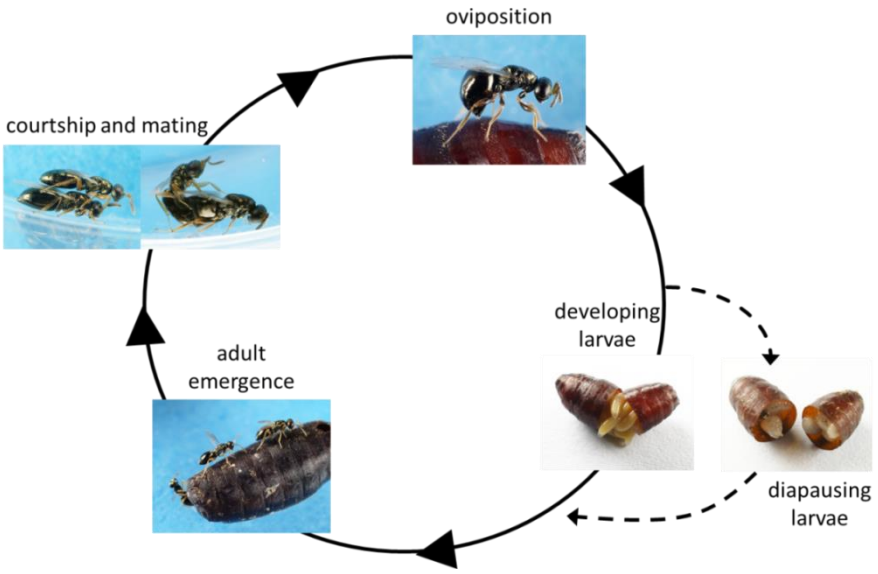

Figure 1.7 Schematic life cycle of *Nasonia vitripennis*. Photos by Peter Komen

***Diapause in* Nasonia**

Nasonia has a facultative diapause that occurs at the fourth larval instar just before pupation and it is induced by the mother which represents the sensitive stage (Saunders, 1965). *Nasonia* adult females are able to produce two types of offspring depending on the specific environmental cues they perceive: the first type develops to the adult stage without arrest while the second type consists of diapausing larvae that require a cold period (winter conditions) to resume development into pupae and

subsequently into adults. The two types of larvae can be distinguished very easily from their morphological features and from the clear arrest of development of the diapausing type.

Photoperiod represents the most important factor inducing diapause in *Nasonia vitripennis* (Saunders, 1965, 1966a). Adult females exposed to short-day conditions produce developing offspring for the first days of their life and switch to the production of diapausing offspring after a threshold number of light:dark cycles. In the past, *Nasonia* photoperiodism has been extensively investigated by Saunders (Saunders, 1962, 1965, 1966a,b, 1969; Saunders *et al*., 1970) who mostly used a strain of *N. vitripennis* that originated from Cambridge (UK) to study diapause response. By exposing individual females to different photoperiodic cycles and scoring diapause response in the offspring, Saunders constructed the photoperiodic response curve for *N.vitripennis* which shows an abrupt change at the critical photoperiod (~15h light) (Fig. 1.8). Diapause is induced at day lengths shorter than the critical photoperiod, and is inhibited when day lengths exceed the critical photoperiod (long-day insect).

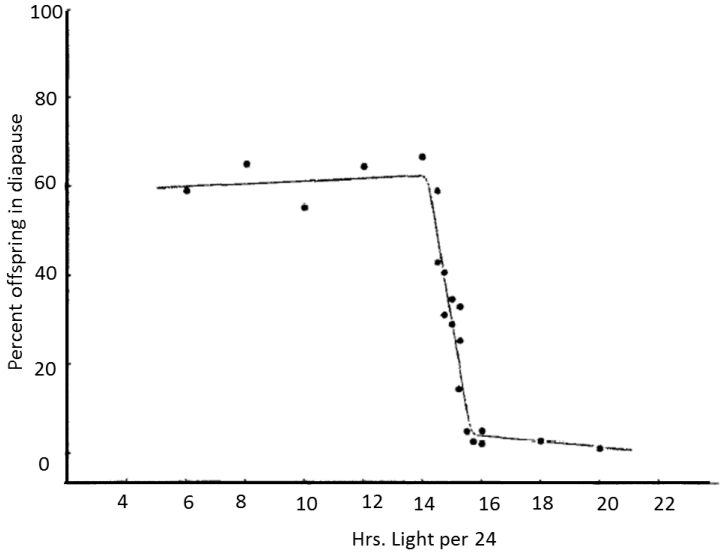

Figure 1.8 Photoperiodic response curve of *Nasonia vitripennis* (Cambridge strain) at constant temperature (18°C) showing abrupt change in diapause around 15 hours of light (Saunders, 1966a)

Besides photoperiod, other environmental factors are known to play a role in induction of diapause in *N. vitripennis* and they have a direct or indirect effect on the modification of the photoperiodic response. For example, the effect of temperature is complex and involves at least two aspects: previous experiments showed that *Nasonia* females kept under the same short day length but different temperatures (between 15°C and 30°C) required the same number of L:D cycles to effect the switch. However, females raised at low temperature lived much longer than females kept under high temperature, resulting in a total number of diapausing offspring higher under low temperature. (Saunders, 1969 and references therein). Daily temperature cycles also affect diapause production in *N.*

vitripennis. Saunders (1973) showed that the thermoperiodic response curve for diapause induction is similar to the photoperiodic response curve with a critical thermoperiod corresponding to around 13 hours of high temperature (23°C) and 11 hours of low temperature (13°C). Females of *N. vitripennis* are therefore able to distinguish short day thermoperiods from long day thermoperiods (Saunders, 1973).

Other factors involved in diapause induction in *N. vitripennis* include food shortage (Saunders, 1966b) and the host species (Saunders *et al.*, 1970). Endogenous factors potentially inducing diapause have also been examined. Maternal age seems to affect diapause production in *N.vitripennis*: Saunders (1962, 1965) showed that old females produce greater proportion of diapausing larvae than young ones, however it was not clear if this effect is directly associated to senescence or if it is rather the manner in which day length, temperature and host deprivation express themselves via egg production (Saunders, 1965).

In one of his studies, Saunders (1965) documented variation in diapause response between two strains: the lab strain originating from Cambridge, UK (52°N) showed a longer critical day length compared to a strain from Massachusetts, US (42°N). The difference in critical photoperiod was about 2 hours suggesting that photoperiodic diapause induction in *Nasonia* varied as a result of local adaptation to specific condition. However, no studies with specific focus on the geographic variation in diapause response in *Nasonia* were conducted before the work presented in this thesis.

AIM OF THIS RESEARCH AND THESIS OVERVIEW

The goal of this PhD project is to investigate the genetic basis of adaptive variation in photoperiodic diapause response, with particular focus on the involvement of clock genes and on the possible link between circadian system and seasonal photoperiodic response. I use a latitudinal cline approach to analyse variation in several aspects of diapause in natural populations of *Nasonia vitripennis* wasps, and I aim to identify the factors involved in establishing the observed clinal variation as a result of local adaptation. The genetic analysis used a combination of QTL analysis and candidate gene approach to identify the genomic regions involved in photoperiodic diapause and to evaluate the role of clock genes on diapause variation. To further explore the role of clock genes in diapause response, I studied the natural variation of genetic polymorphism in the clock gene *period* which was found to be associated with variation in diapause response. Finally, I attempt to explain the variation in circadian rhythmicity in natural populations of *Nasonia vitripennis.*

Chapter 2 of this thesis describes the different aspects of photoperiodic diapause response in natural populations of *Nasonia vitripennis* in Europe. I mainly focus on the sensitive stage of diapause induction (adult female) and show the existence of a latitudinal cline in the critical photoperiod for diapause induction and in switch point, measured as the maternal age at which females switch from the production of non-diapausing offspring to the production of diapausing offspring under specific photoperiodic regimes. The switch point corresponds to the number of photoperiodic cycles that need to be experienced by the adult

female before diapause is induced in the offspring. This number decreases towards northern latitudes where females have a faster response compared to southern populations. I discuss the adaptive value of such latitudinal cline and the relevance of the sensitive stage as target for natural selection leading to the establishment of the cline.

In **Chapter 3**, I study the modes of inheritance of photoperiodic diapause induction in *N.vitripennis* through crosses between two isofemale lines from the extreme northern and southern locations of the sampled sites and analyse diapause response in subsequent generations. I show that photoperiodic diapause is a female induced trait and the maternal nuclear genes are mostly responsible for variation in response, with no cytoplasmic effects. The QTL analysis, where SNPs in candidate clock genes were used as markers, revealed two main genomic regions involved in photoperiodic diapause. Interestingly, the *period* locus corresponds to the main QTL peak, suggesting a functional role for the *period* gene in diapause.

The investigation of *period* and its role continues in **chapter 4**, where I show that sequence polymorphism in exons of this gene in the northern and southern lines is associated with variation in phenotypic diapause response. Two main alleles were identified and their frequency was studied in lines from all clinal populations. I observed latitudinal variation in the allele frequency of *period* which correlates with latitudinal variation in photoperiodic diapause. The results of chapters 3 and 4 suggest a link between seasonal photoperiodic response and circadian clock as there is an association between diapause variation and *period* gene. Since *period* is a clock gene regulating circadian systems, I

hypothesize that a corresponding natural variation in properties of the circadian clock will be present in *N. vitripennis* clinal populations. Hence, in **chapter 5** I describe the natural variation in circadian rhythmicity, measured as free running period in locomotor activity of wasps under constant conditions. I also investigate the level of activity of the two northern and southern selected lines under different environmental conditions. The results show that lines differ in specific aspects of circadian activity.

In the final **chapter 6** I merge the results of my research, summarize the current knowledge about photoperiodism in *Nasonia vitripennis* and propose avenues for further research

Chapter 2

Adaptive latitudinal cline of photoperiodic diapause induction in the parasitoid *Nasonia vitripennis* in Europe[2]

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ABSTRACT

Living in seasonally changing environments requires adaptation to seasonal cycles. Many insects use the change in day length as a reliable cue for upcoming winter and respond to shortened photoperiod through diapause. In this study we report the clinal variation in photoperiodic diapause induction in populations of the parasitoid wasp *Nasonia vitripennis* collected along a latitudinal gradient in Europe. In this species, diapause occurs in the larval stage and is maternally induced. Adult *Nasonia* females were exposed to different photoperiodic cycles and lifetime production of diapausing offspring was scored. Females switched to the production of diapausing offspring after exposure to a threshold number of photoperiodic cycles. A latitudinal cline was found in the proportion of diapausing offspring, the switch point for diapause induction measured as the maternal age at which the female starts to produce diapausing larvae, and the critical photoperiod for diapause induction. Populations at northern latitudes show an earlier switch point, higher proportions of diapausing individuals and longer critical photoperiods. Since the photoperiodic response was measured under the same laboratory conditions, the observed differences between populations most likely reflect genetic differences in sensitivity to photoperiodic cues, resulting from local adaptation to environmental cycles. The observed variability in diapause response combined with the availability of genomic tools for *Nasonia vitripennis* represent a good opportunity to further investigate the genetic basis of this adaptive trait.

INTRODUCTION

In temperate and polar zones, light-dark periods and temperature show seasonal fluctuations. Species living in such areas synchronize their life cycle with annual cycles of environmental factors in order to optimally use the resources available during the favourable season for their growth and reproduction and to survive during harsh conditions. Organisms cope with seasonal change in a variety of ways, including different types of physiological, behavioural and developmental adaptations. Such long-term adaptations usually involve genetic changes, combined with plastic responses and physiological adjustments that are characteristic of short term responses (Gienapp *et al.,* 2008; Hoffmann & Sgró, 2011).

Many insect species spend the unfavourable season in a physiological state of dormancy called diapause mediated by neuro-hormonal signals, in which development is arrested, metabolic activity reduced and resistance to environmental challenges increased (Tauber *et al*., 1986). The developmental stage at which diapause occurs ranges from embryo to adult, but each species has a genetically determined and specific expression of the diapause syndrome (Tauber *et al*., 1986; Danks, 1987), Commonly, insects show facultative diapause in which specific developmental stages are sensitive to environmental stimuli. Only when cues for approaching seasonal change (for example upcoming winter) are perceived, insects respond adaptively by entering diapause (Tauber *et al*., 1986). The evolutionary significance of the diversity in diapausing stages among insects has hardly been addressed. This requires comparative studies in connection with related life history traits in a variety of insect

species.

Various environmental factors can induce diapause response (Tauber *et al*., 1986), but the majority of species living in temperate zones use the change in daily light:dark cycles (photoperiod). This is the most reliable cue for seasonal change as it remains constant over geological time (Bale & Hayward, 2010) and is correlated with other factors such as temperature, moisture and food availability (Tauber *et al*., 1986). Photoperiod changes gradually and consistently along a latitudinal gradient, and insects at different latitudes have evolved specific responses to the prevailing photoperiods. The critical photoperiod, corresponding to the day length at which 50% of individuals in a population enters diapause, is longer towards higher latitudes (Kurota & Schimada, 2003; Wang *et al.,* 2011).

Despite the large number of studies on latitudinal variation of diapause response, its genetic regulation and the evolutionary mechanisms behind it are still poorly understood. In order to get insight into these mechanisms, it is important to investigate each aspect of diapause and particularly the genetic variation underlying variation in the sensitive stage during which the information from the environmental cues is perceived. The fruit fly *Drosophila melanogaster* has been extensively used as a model species for studying the molecular basis of adult reproductive diapause and the silk moth *Bombyx mori* for induction of egg diapause (Schiesari *et al.*, 2011). These studies show that information from the environment is processed by specific brain regions that stimulate hormone production, which in turn activates pathways for signal transduction, development and stress resistance (Nelson *et al*., 2010).

Natural selection can act on different levels of the pathway leading to diapause. For example, the observed geographic variation in diapause induction can be determined by genetic variation for detection of environmental cues. Alternatively (or simultaneously), selection can act on hormone production or on the regulation of signal transduction that induce the diapause response. In order to understand the genetic basis of adaptation to seasonal environmental cycles it is crucial to elucidate those aspects of diapause that constitute the target of natural selection and vary in relation to the environment. This requires insect species with robust and clear photoperiodic responses, distinct sensitive and responsive stages and well developed genomic tools (Emerson *et al*., 2009).

The haplodiploid Hymenopteran genus *Nasonia* is emerging as new model system in evolutionary biology (Beukeboom & Desplan, 2003; Werren *et al*., 2010). *Nasonia* are small gregarious parasitoid wasps that parasitize the pupal stage of various blowfly species. There are four closely related species, of which *Nasonia vitripennis* has a cosmopolitan distribution and therefore can cope with a wide range of climatic conditions (Darling & Werren, 1990). *Nasonia* has a facultative diapause which occurs at the fourth larval instar just before pupation and which is well defined and easy to measure (Saunders, 1965). Interestingly, it is induced by the adult female which therefore represents the sensitive stage. In general, when adult females are exposed to short-day conditions (short photoperiod), they initially produce normal developing larvae and switch to production of diapausing larvae after exposure to a critical number of light:dark cycles. Under long photoperiodic conditions the switch occurs later or not at all. The larval diapause is very strong and

remains until specific environmental conditions, such as increasing temperature, induce further development (Saunders 1962, 1966a). Saunders used *N. vitripennis* extensively for studying the mechanisms of photoperiodism and proposed that photoperiodism consists of two separate components: a photoperiodic timer which measures the length of the day (or night), and a photoperiodic counter which counts the number of cycles. The counter uses the information from the timer to elicit the response after a threshold number of photoperiodic cycles has been reached (Saunders 2002). Studying geographic variation of this threshold level during the sensitive stage will shed light onto the proximate mechanisms underlying diapause variation and the genetics of seasonal adaptation. In this respect, *Nasonia* represents an excellent model system since it provides the opportunity to study variation in the perception of environmental conditions during the sensitive stage (adult female) and how this is translated into different response in the offspring.

Here, we describe the latitudinal variation in photoperiodic induction of diapause using seven *Nasonia vitripennis* populations collected along a latitudinal gradient in Europe, as a first step towards unravelling the genetic architecture of this important life-history trait and its variation. For all populations, we measured the critical photoperiod (expression of the photoperiodic timer), the number of photoperiodic cycles required for the induction of diapause (photoperiodic counter) and the overall proportion of diapausing offspring produced during the entire life of adult females exposed to different photoperiodic regimes. We link these results to molecular data on genetic divergence of populations to evaluate the role of selection in maintaining the observed variation.

MATERIAL AND METHODS

Field collection

In summer 2009, *Nasonia* wasps were collected from seven locations along a latitudinal gradient in Europe from northern Finland to Corsica, covering a range of about 23 degrees (Fig. 2.1. OUL: 65°3'40.16”N, 25°31'40.80"E; TUR: 61°15'40.53"N, 22°13'23.96"E; LAT: 56°51'22.56"N, 25°12'1.38"E; HAM: 53°36'23.62"N, 10°10'17.74"E; SCH: 50°19'56.10"N, 9°30'47.00"E; SWI: 46°44'9.14"N, 7°6'57.34"E; COR: 42°22'40.80"N, 8°44'52.80"E). Wasps were collected from bird nests in nest boxes. The nest boxes were mainly used by great tits (*Parus major*), blue tits (*Parus caeruleus*) and flycatchers (*Ficedula hypoleuca*). At least two sampling sites, each including several nest boxes, were visited at each location in order to increase the diversity of samples within the same geographical location. Wasps were collected in three ways. The main sampling technique consisted of removal of nests at least 5 days after the birds had fledged and subsequent dissection for fly pupae that might have been parasitized. The second sampling methodology involved the use of baits consisting of mesh bags with approximately 25 laboratory-raised fly pupae (*Calliphora spp*.) that were placed in nest boxes for a few days to attract *Nasonia.* They were subsequently taken to the laboratory for further development and checked for wasp emergence. Thirdly, adult wasps could also be collected directly in the field from the nest material or on the baits. Table 2.1 provides an overview of the total number of nest boxes that were sampled at each location. Wasps collected within a nest box may be genetically related because a single female can parasitize several

fly pupae within one nest, although most nests are colonized by multiple unrelated founding females (Grillenberger *et al*., 2008).

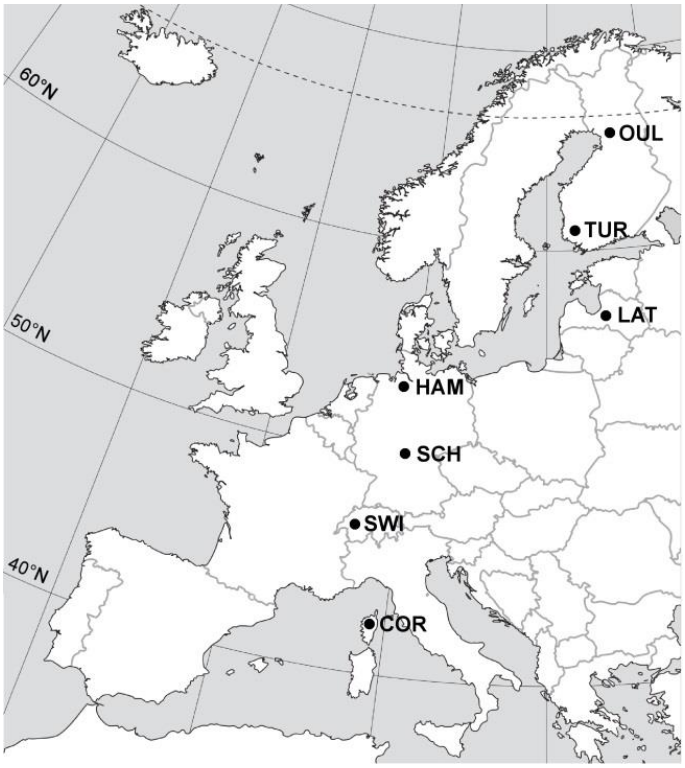

Figure 2.1: Map of sampling locations in Europe. From North to South: Finland, Oulu (OUL); Finland, Turku (TUR); Latvia (LAT); Germany, Hamburg (HAM); Germany, Schlüchtern (SCH); Switzerland (SWI); France, Corsica (COR)

Establishment of isofemale lines

Isofemale lines were established from females collected directly from the field, or from nest and bait emergences. The pool of natural pupae obtained from a single nest box or bait was maintained in a vial until

emergence of flies or wasps. Flies were discarded, while emerged wasps were left for one day in order to allow mating within the patch. Single females were subsequently isolated in a plastic vial and supplied with hosts (*Calliphora spp.* pupae). As *Nasonia* females typically mate once, a maximum of three different alleles per gene will segregate in each line because of haplodiploidy, but this number may be reduced due to mating among relatives (Grillenberger *et al*., 2008). In some cases, multiple lines had to be established from one nest box when not enough females were available because too few nest boxes or baits yielded wasps. Isofemale lines were maintained in mass culture vials at diapause-preventing conditions (long photoperiod, temperature = 20-25°C). Throughout this chapter, the term 'population' will be used for the pool of isofemale lines established from each location.

Some wild-caught females from northern latitudes (OUL and TUR) produced only diapausing offspring after field collection and therefore could not be used immediately in the first experiment. These lines were established as 'diapause lines' and maintained under diapause conditions (temperature = 5°C, constant darkness). After a few months they were transferred to 20°C and light:dark (LD) cycle 18:6 to break diapause and subsequently used in a second experiment (see below) to determine their diapause response.

Table 2.1: Summary of field collection showing the number of nest boxes that yielded wasps in each sampling location and isofemale lines used in the experiment. For each locality the number of used nest boxes is given and the number of used isofemale lines established from nest material, baits and wild-caught females in those nests. Lines used in the second experiment are shown in the panel on the right. Location abbreviations are explained in Fig. 2.1

	Location	COR	SWI	SCH	HAM	LAT	TUR	OUL	TUR	OUL
Field work	Nest boxes inspected	129	87	106	60	70	55	84		
	Nest boxes that yielded wasps*	23	12	6	7	11	13	29		
Experiment	Nest boxes used	8	12	6	7	11	11	19	5	15
	Lines from nest material	0	22	22	18	20	6	10	0	0
	Lines from baits	0	2	0	6	4	5	0	0	0
	Lines from wild-caught females	25	2	0	2	2	10	16	6	18
	Total	25	26	22	26	26	21	26	6	18

* from natural host puparia, baits or as adult female individuals

Photoperiodic induction of diapause

Twenty-one to 26 isofemale lines per location were used to measure diapause response under different photoperiods. Given that the main goal of this experiment was to investigate latitudinal variation in photoperiodic response rather than variation within local geographic populations, the number of independent lines was maximized and lines were considered as replicates within a location. The details about number of isofemale lines and their source for each location are given in Table 2.1. Individuals from the first or second generation after field collection were used. Prior to their use, they were kept under standard conditions (25±1°C, LD 18:6) in mass cultures. Newly emerged individuals were allowed to mate among

themselves (siblings) for one day. Single females were subsequently placed in cotton-plugged 60 mm x 10 mm polystyrene tubes with two hosts. Wasps were distributed over eight incubators corresponding to the eight treatments used in the experiment, resulting in a sample of 21-26 individuals per location in every condition. Each treatment consisted of a constant photoperiod (hours of light in 24 hours). The following treatments were applied: LD 8:16, LD 10:14, LD 12:12, LD 13:11, LD 14:10, LD 15:9, LD 16:8, LD 18:6 (Light intensity: 100-200 lum/sqf). All treatments were at constant temperature (T=20±1°C) and humidity (50-55% RH).

Females were exposed to the treatments for their entire life and the two hosts were replaced every other day. Parasitized hosts were transferred to a new vial and cultured at 20±1°C and constant light. This ensured standardized developing conditions of offspring from all individuals in all treatments. Females were re-hosted until death providing additional data about adult longevity under different LD regimes.

Diapause scoring

Diapause in *Nasonia* occurs at the fourth instar larval stage. Typically, females produce normal developing larvae at the beginning of their life and switch to production of diapausing larvae later in life after exposure to a certain number of LD cycles. Diapausing larvae arrest their development and resume it only after having been kept under diapause-maintaining conditions for at least two months (temperature ~4°C, constant darkness). Normal developing larvae emerge from the host as adults after 21 days at 20°C. Thus, diapause can easily be determined by opening the hosts after 21 days and scoring for presence of larvae.

A total of 20987 broods were scored for diapause. Diapause was measured as a binary trait: each set of two hosts provided in a 2-days interval was scored as 'diapause' when only diapausing larvae were present or 'no diapause' when only adult offspring emerged. In the case of mixed broods, if 50% or more individuals were diapausing larvae, they were counted as 'diapause broods', otherwise as 'non-diapause broods'. Mixed broods were rare and typically only occurred in one or two hostings around the switch point. Very few hosts were empty or yielded flies (non-parasitized) and were excluded from the dataset.

For each female, two parameters were measured: *the production of diapause offspring* which corresponds to the proportion of diapausing broods relative to the total number of broods in her life and the *switch point* measured as the maternal age at which the female switches from producing non-diapausing to diapausing offspring during the sequential hosting (i.e. the required number of days of exposure to a particular LD regime for the occurrence of the switch). For each population, the *critical photoperiod* for diapause induction was estimated as the photoperiod corresponding to 50% of diapause response (see statistical analysis). The correlation between all parameters and latitude was measured.

Photoperiodic induction of diapause in 'diapause lines' from northern locations

In a second experiment, additional isofemale lines (Table 2.1) were used that had been established as 'diapause lines' right after collection (see above). These lines were from the two most northern locations and needed to be added to the analysis to prevent a biased photoperiodic

response measurement. Females were taken out of diapause after two months storage at 4°C and cultured for an extra generation under standard conditions (25±1°C, LD 18:6). The experimental setup was the same as the first experiment, except that only five LD treatments were applied (LD 13:11, LD 14:10, LD 15:9, LD 16:8, LD 18:6) and females were re-hosted every other day until day 20 of adult life.

Effect of laboratory culture on photoperiodic diapause induction

Our experiments to determine the natural variation of photoperiodic diapause response were performed using individuals from the first or second generation after field collection in order to keep the genetic make-up of the different isofemale lines as close as possible to the original state. In a separate experiment, a possible effect of adaptation to lab condition was investigated by re-testing a number of isofemale lines from three locations (COR, HAM and OUL) after having been maintained for 13-14 generations in the lab. In addition, replicates of the same lines that had been kept in diapause from the second or third generation after field collection (for about 10 months) served as controls. After synchronization and standardization of culturing conditions (25±1°C, LD 18:6) for two generations, adult females were exposed to five LD treatments (LD 13:11, LD 14:10, LD 15:9, LD 16:8, LD 18:6) and re-hosted every other day for the first 20 days of adult life. Diapause was subsequently scored as previously described using 10 to 16 lines per location.

Microsatellite genotyping for analysis of population differentiation

The wild-caught adult females and those used to establish the isofemale

lines were stored in 70% Ethanol at -20°C prior to DNA extraction and molecular analysis. Genomic DNA was isolated from individual females using a standard high salt-chloroform protocol (Maniatis *et al*., 1982). Fifteen to 26 individuals per location originating from different nest boxes within each location were selected. Genetic differentiation between populations was established using eleven polymorphic microsatellite markers (Nv26, Nv107, Nv118, Nv200, Nv205, Nv229, Nv301, Nv303, Nv319, Nv320, Nv322) (Beukeboom *et al*., 2010; Pannebakker *et al.,* 2010; Beukeboom, unpublished) that were amplified using the Qiagen multiplex PCR kit according to manufacturer's recommendations (PCR profile: 15 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 1.5 minutes at T_A = 57°C and 1 minute at 72°C, followed by 45 minutes at 72°C). The length of the amplified fragments was determined using the Applied Biosystems 3730 DNA Analyzer and analysed using GENE MAPPER v4.0 (Applied Biosystems, Carlsbad, CA, USA).

Statistical analysis

Statistical analysis of all data on photoperiodic diapause induction was performed using the R statistical software (R Development Core Team 2012). The general technique for building up a statistical model followed the standard model simplification procedure starting from full model with all possible factors and interactions and proceeding by removing non-significant explanatory factors. The fit of the models was assessed after comparing the likelihood of different models with Chi-squared test. ***Proportion of diapause production.*** To analyse the data on variation in lifetime proportion of diapause we used a generalized linear mixed effect

model (R package *lme4*). The response variable is a matched pair of counts of non-diapause broods and diapause broods which is interpreted as the proportion of broods that were in diapause for each female. Location and treatment were included as fixed explanatory variables, the random effects were nested as follow: nest box (from which each isofemale line was established) nested within the location nested within LD treatment using the binomial error distribution. The correlation between latitude and proportion of diapause was analysed with a series of mixed effect logistic regressions for each treatment independently. In these models, latitude was fitted as fixed continuous explanatory variable and nest box nested in location as the random effect. The error distribution is binomial and the link function is logit. The validity of the model was assessed through comparison between the model with latitude and the null model with only random effect (likelihood ratio test). The odds ratios are calculated from the coefficients estimated in the model.

Switch point for diapause induction. In the second analysis, we investigated the switch point for diapause induction in different treatments for different populations. Survival analysis was used to analyse the time of switch as response variable. This is expressed as the maternal age at the switch point which corresponds to the number of LD cycles experienced by the adult female before the switch would occur. Data were analysed using Cox proportional hazard mixed effects model (package *coxme* in R) and data were censored for individuals that did not live long enough to reach the switch point. In the first model, location and treatment were the fixed effects, the random effects were specified as above. The effect of latitude on switch point within each treatment was

analysed separately to check for a correlation between latitude and switch point. Hazard ratios were obtained from these models as estimates of the difference in the rate of switching to diapause for every unit of the predictor variable 'latitude' (one degree unit). A positive value of the hazard ratio means that the risk of switching to diapause per unit of time increases for every degree of latitude, which indicates that the chance of inducing diapause is early in life (higher rate) at higher latitudes and later in life at low latitudes. This in turn results in a negative correlation between switch point and latitude.

Survival analysis (Cox proportional hazard mixed effects model) was also applied in a similar way to test the effect of treatment and location on longevity of wasps. In this case, there were no censored data as all wasps eventually died. Correlation between latitude and life span was also tested with the same type of survival analysis.

Critical photoperiod for diapause induction. For the estimation of the critical photoperiod of each population, we focused mainly on 10-day-old females because this age point coincided with the largest variation in response between populations. A mixed effect logistic regression model was fitted for specific age points, considering diapause as a binary response variable, treatment and location as fixed effect, and nest box nested within location nested within treatment as random effects. The critical photoperiod for each population was established after fitting an incidence function model (generalized linear model with binomial error) based on diapause incidence as function of photoperiod which yielded the photoperiod corresponding to 50% of diapause response. The diapause incidence is defined as the proportion of females of a given population

producing diapausing offspring at a certain age and under specific condition.

The correlation between critical photoperiod and latitude was determined with linear regression analysis (*lm* in stats package in R) between estimated critical photoperiod and mean latitude of origin of each population. Data on photoperiodic diapause induction in northern lines subsequently added to the dataset and data on diapause response of lines maintained in the laboratory for several generations were analysed using the same types of statistical methods.

Population differentiation analysis. Population structure of *N.vitripennis* European populations was determined with the software Fstat (Goudet, 2001) from 11 microsatellite markers. Gene diversity was estimated per locus and per population using an unbiased estimate of genetic variability (gene diversity, Nei, 1987) as implemented in Fstat. Single and multilocus F_{ST} values were calculated according to Weir and Cockerham (1984) and SE were obtained after jackknifing over populations or loci. The same procedure was used to obtain pairwise F_{ST} values.

Pairwise tests of differentiation were performed following G-statistic and significant values are considered at the nominal level of 0.05 after Bonferroni correction. The pairwise F_{ST} values and the approximate geographic distance (km) between geographical locations were used for the isolation by distance analysis performed with a Mantel test for matrix correlation.

RESULTS

Latitudinal cline for proportion of diapause production

Diapause was induced in *N. vitripennis* larvae after exposure of females from the maternal generation to different LD regimes, confirming that photoperiod represents an important diapause-inducing factor. All tested isofemale lines produced diapausing larvae under at least one of the applied LD regimes. In general, the overall production of diapausing broods was higher for females exposed to short photoperiods and decreased with longer photoperiods (Fig. 2.2; mixed effect logistic regression model, effect of treatment: χ^2 = 42.43, P = 4.29e-07). There was also a clear effect of geographical origin (mixed effect logistic regression model, effect of location: χ^2 = 142.22, P < 2.2e-16). In addition, for all LD regimes a positive correlation was found between latitude and proportion of diapausing broods (mixed effect logistic regression models, all P values are < 0.05, odds ratios are given in Figure 2.2). This indicates a latitudinal cline for photoperiodic diapause induction in *N. vitripennis*. As the diapause production was measured under the same experimental conditions, the observed variation in response reflects genetic differences in sensitivity to specific photoperiods between different populations.

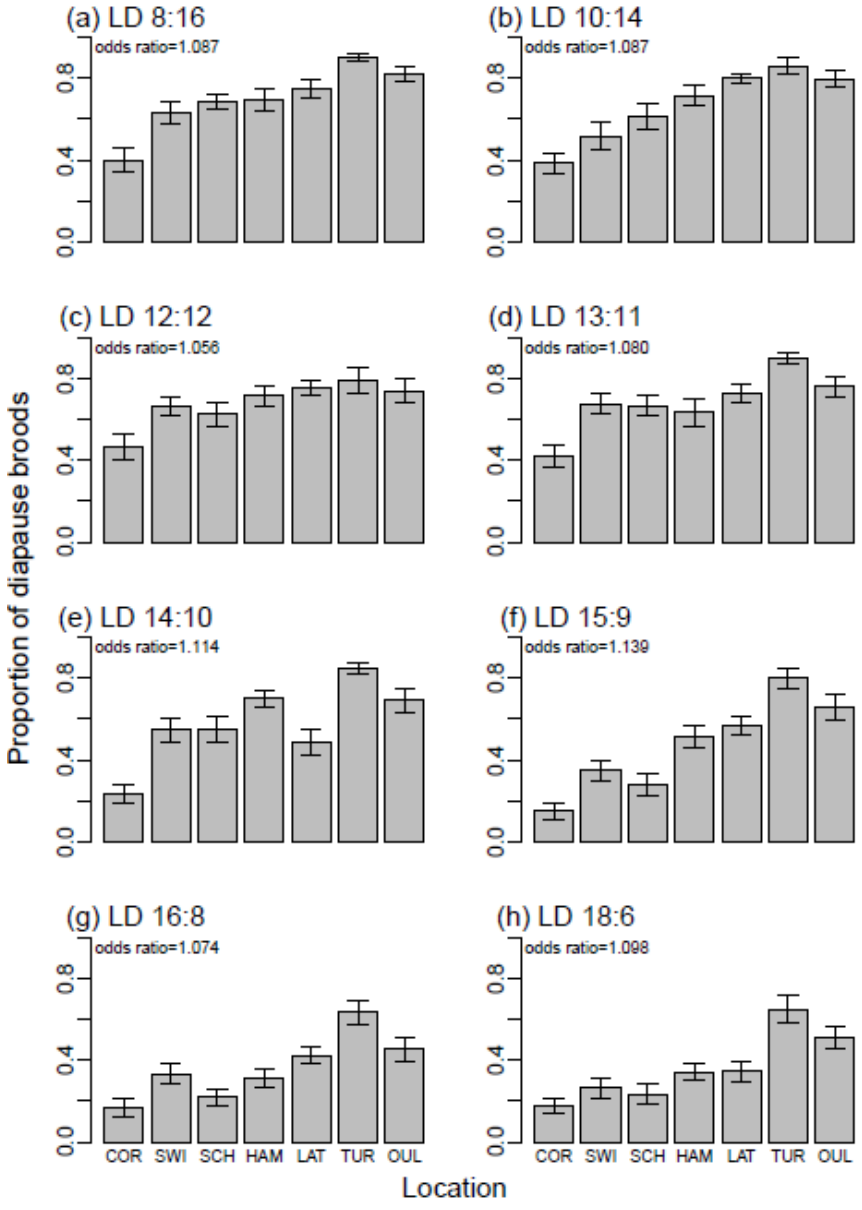

Figure 2.2: Proportion of diapausing broods relative to the total number of broods produced by females (mean ± SE) from seven geographic locations under applied LD treatments. The odds ratios obtained from the coefficients estimated by the logistic regression model are given in each panel. The sample size is 21-26 individuals in each location and treatment.

Latitudinal cline for switch point (photoperiodic counter)

Adult *N. vitripennis* females exposed to experimental LD regimes initially produced normal developing offspring and switched to the production of diapausing offspring later in life. For each female, the switch is pronounced and rapid; broods containing a mixture of diapausing larvae and non-diapausing larvae are rare and occur only for one or two days around the switch.

Eighty-five percent of all tested females lived long enough to pass the switch point. For COR, the most southern population, 69 out of 200 females did not switch, when exposed to long photoperiods and also showed the shortest life span. The required day number for the switch was dependent on the applied LD regime (Fig. 2.3) and the geographical location of the population. For all populations, adult females under short photoperiods switched to the production of diapause broods earlier compared to long photoperiods. Additionally, for each regime, individuals from southern latitudes switched later than those from northern latitudes, indicating a correlation between latitude and switch point. The effect of latitude was more pronounced at intermediate photoperiods (LD 14:10 and LD 15:9) corresponding to the largest variation in switch point across locations. This is reflected by a significant effect of the interaction between location and treatment (survival analysis, Cox mixed effects model, effect of treatment x latitude: χ^2 = 77.03, P = 0.0007). This correlation between latitude and switch point was supported by a nonparametric survival analysis, using a series of Cox models for each treatment separately. In all treatments, latitude and switch point were negatively correlated (survival analysis, Cox mixed effects models, all P

values < 0.05, hazard ratios are given in Fig. 2.3) meaning that southern individuals switch later in life compared to northern individuals.

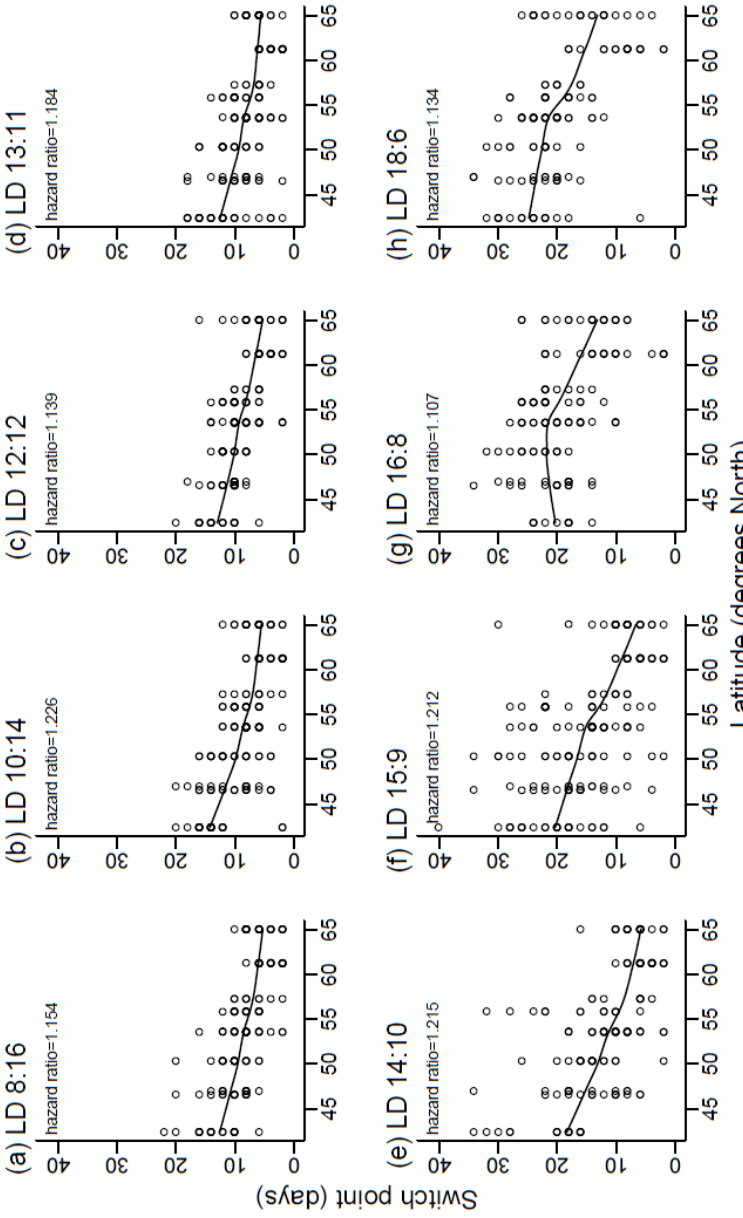

Figure 2.3: Switch point for diapause induction of females from different latitudes exposed to different LD treatments. Maternal age at switch point is shown for those females that reached the switch. The fitted curves in each panel are computed by Loess smoothing. Hazard ratios obtained from the statistical model are given in each panel. The sample size is 21-26 individuals in each location and treatment

In order to investigate whether the observed cline in switch point is caused by latitudinal differences in female life span, we measured adult longevity at 20°C. There was no effect of LD treatment (survival analysis, Cox mixed effects model, effect of treatment: $\chi^2 = 5.37$, $P = 0.61$) meaning that photoperiod itself does not affect life span in *N. vitripennis*. Considerable variation, however, was found between individuals from different locations (Fig. 2.4): mean adult longevity ranged from 26.3 (COR) to 36.2 (LAT) days and there was a significant effect of location (survival analysis, Cox mixed effects model, effect of location: $\chi^2 = 36.526$, $P = 2.18e\text{-}06$). Although populations differed in their mean life span, there was no correlation between longevity and latitude (survival analysis, Cox mixed regression model, effect of latitude: $\chi^2 = 0.99$, $P = 0.32$) indicating that the observed differences between locations are not due to the latitudinal cline. These results imply that the observed variation in switch point is not affected by variation in longevity.

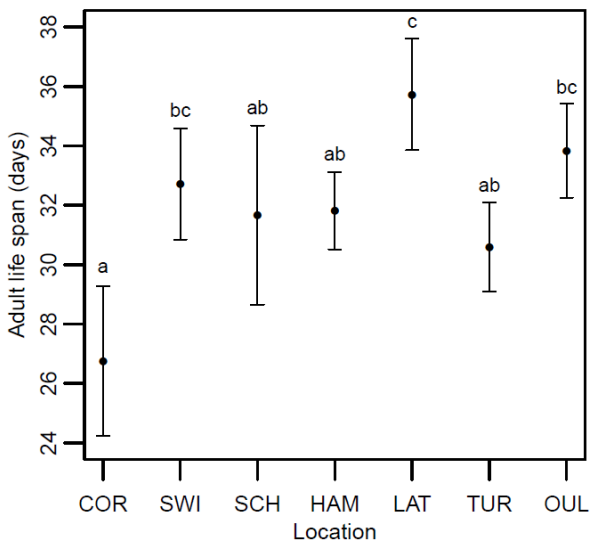

Figure 2.4: Life span of females from the seven locations (mean ±SE). Data from different LD treatments are pooled (there is no significant effect of treatment) which increased the sample size in each location to 160-208 individuals per location. Letters indicate significant differences (*post hoc* multiple comparison for survival analysis, $P < 0.05$)

Latitudinal cline for critical photoperiod (photoperiodic timer)

The effect of photoperiod on diapause induction for 10-day-old females was chosen for comparison of critical photoperiod as this time point showed the largest variation in response between locations. For the construction of photoperiodic response curves (Fig. 2.5), the incidence of diapause is defined as the percentage of individuals in a given population that switched to the production of diapausing larvae under a certain condition. In line with the results shown in the first section, geographic origin and LD regimes had an effect on diapause induction (mixed effect logistic regression model, effect of location: χ^2 = 140.06, P < 2.2e-16; effect of treatment: χ^2 = 42.81, P = 3.64e-07). Overall, diapause induction was high in populations from northern locations and decreased in more southern populations.

The photoperiodic response curves for the different populations have similar shapes reflecting high diapause incidence at short photoperiods, low incidence at long photoperiods and an abrupt change at intermediate photoperiods (in the range between LD 13:11 and 16:8). The response curve for the three most southern populations for LD 10:14 deviates from the expected trend and showed an unexpected low diapause response. We explain this as an experimental incubator effect, in which deviant temperature and/or humidity delayed diapause in the three most southern populations. As these populations generally have weaker response compared to northern populations their switch point may be more sensitive to small changes in environmental conditions.

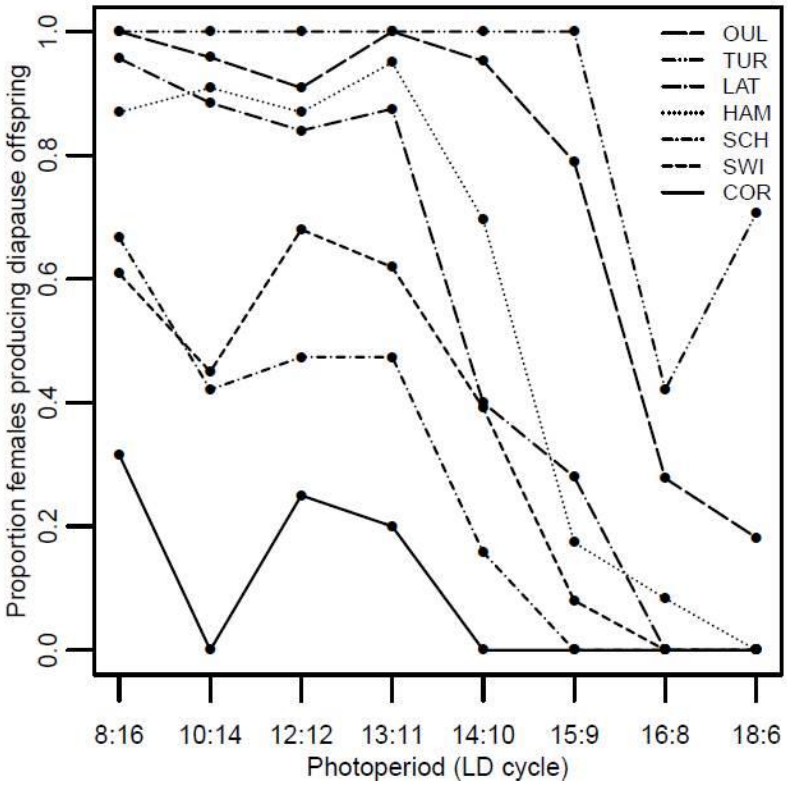

Figure 2.5: Photoperiodic response curves for diapause induction in 10-day-old *Nasonia vitripennis* females from seven geographic locations. The sample size is 21-26 individuals in each location and treatment.

The critical photoperiod was estimated for each population after fitting an incidence function model based on diapause incidence as function of photoperiod, excluding the LD 10:14 in all populations in order to obtain a better fit of the model. As not all populations reached 100% of diapause incidence under the experimental conditions at the chosen age point, the critical photoperiod was defined as the photoperiod at which diapause occurred in 50% of the females that produced diapausing offspring. The estimated critical photoperiods ranged from 10.5 (COR) to 18.0 hours

(TUR) and showed positive correlation with the mean latitude of population origin. This reflects a clear latitudinal cline for critical photoperiod inducing diapause in *N.vitripennis* (Fig. 2.6).

To confirm the conclusions for 10-day old females, the critical photoperiod in each population was estimated using data from day 16. Figure 6 shows the linear regressions between latitude and estimated critical photoperiod using data from 10-day-old (linear regression: F = 18.8, P = 0.007, estimated slope: 0.286±0.066, adjusted R^2 = 0.75) and 16-day-old females (linear regression: F = 12.49, P = 0.016, estimated slope: 0.25±0.069, adjusted R^2 = 0.66). The diapause incidence for 16-day-old females reached 100% in all populations in short photoperiods and estimated critical photoperiods are longer for all populations. However, the same pattern as for 10-day old females is evident, the critical photoperiod increases to the north.

The estimation of critical photoperiod for the TUR population was more complicated due to the high incidence of diapause in this population also at very long photoperiods (LD treatment 18:6). In this regard, the TUR population deviates substantially from the latitudinal cline showing a critical photoperiod longer than the OUL population.

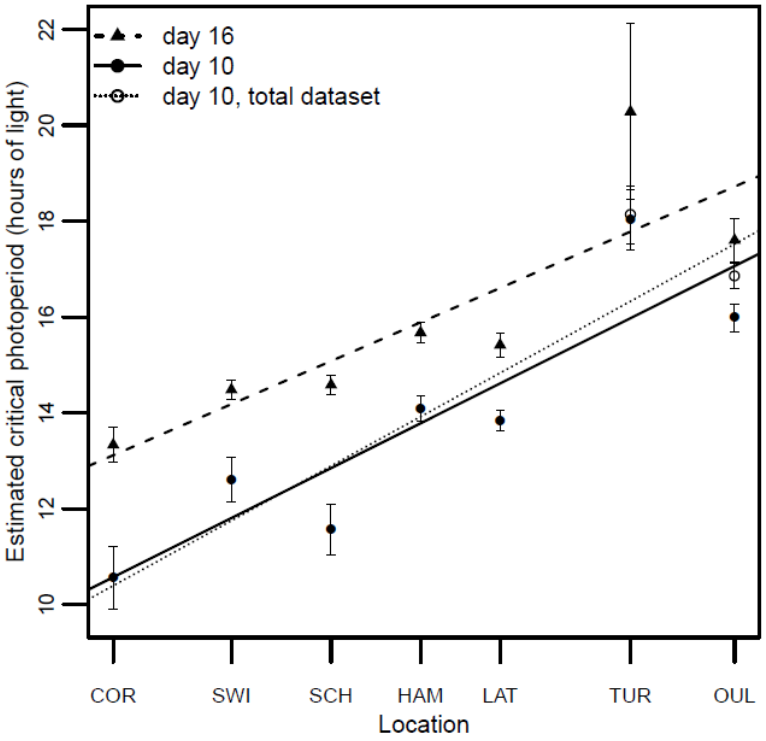

Figure 2.6: Linear regressions between estimated critical photoperiod (±SE) and latitudinal origin of populations for 10 and 16-day-old females (sample size is 21-26 individuals in each location). The difference between the regression lines for 10-day-old females is due to the inclusion or exclusion of high diapause lines from the two northern populations (see text for details). When the high diapause lines are included the sample size for TUR is 27 and for OUL is 44 individuals. The seven locations are spaced along the x-axis according to the mean latitude (degrees) of origin.

Additional data on 'high diapause' northern lines

A number of isofemale lines from the most northern locations (OUL, TUR) could not be tested in the first experiment because they immediately produced diapausing larvae after collection. After emergence from diapause, females were exposed to five LD regimes for the first twenty days of their adult life. All surviving females in all treatments reached the

switch point for diapause induction before day 20, most of them switched before day 10, indicating a very fast diapause response typical of northern populations. The switch point was not significantly different for the individuals from the two locations (survival analysis, Cox mixed effects model, effect of location: χ^2 = 1.00, P = 0.314). The LD regime had a significant effect (survival analysis, Cox mixed effects model, effect of location: χ^2 = 13.06, P = 0.008): the switch occurred later under longer photoperiods in accordance with the data for these populations from the previous experiment.

We estimated a new critical photoperiod for the most northern populations, combining data from 10 day-old-females from the first and the second experiment and re-calculated the linear regression between latitude and critical photoperiod to test for a latitudinal cline in critical photoperiod (linear regression: F = 26.14, P = 0.004, estimated slope: 0.314±0.061, adjusted R^2 = 0.81) (Fig. 2.6). In the new dataset the estimated critical photoperiod of the OUL population was 16.9 hours, about 0.9 hours (54 minutes) longer than the critical photoperiod estimated with the first dataset. This indicates that the OUL lines used in the first experiment likely constituted a biased sample because the lines with highest proportion of diapausing offspring and long critical photoperiods were not yet included. The new estimated critical photoperiod for the TUR population did not differ from the one estimated using the first dataset and it was quite long, again consistent with a very northern type of diapause response.

Effect of laboratory culture on photoperiodic diapause induction

The photoperiodic response after maintenance in the lab for 5-6 generations and for 13-14 generations after field collection was compared to the initial response. After 13-14 generations of lab maintenance still a clear difference between locations was observed as well as of the effect of treatment (mixed effect logistic regression model for data of 10-day-old females, effect of location: χ^2 = 115.66, P < 2.2e-16; effect of treatment: χ^2 = 30.85, P < 3.29e-06) (Fig. 2.7). The northern population (OUL) showed high diapause incidence and long critical photoperiod, the southern location (COR) low diapause incidence and very short critical photoperiod. The response of the HAM population was intermediate. The persistent variation in diapause shows that photoperiodic response was not affected by lab culturing (at least for 13-14 generations) (mixed effect logistic regression model for data of 10-day-old females, effect of lab maintenance: χ^2 = 3.65, P = 0.16) and confirms that the variation is based on genetic differences.

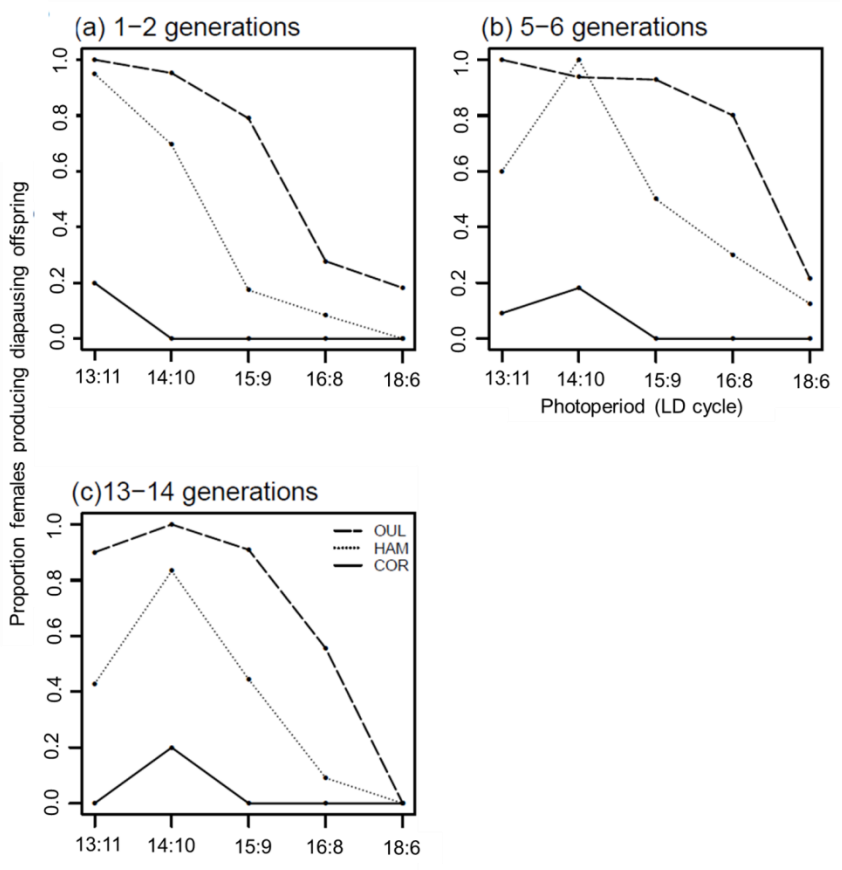

Figure 2.7: Effect of laboratory culture on photoperiodic diapause induction in three populations (COR, HAM, OUL). Photoperiodic response curves are constructed with data from 10-day-old females which were selected from lines maintained in the laboratory for 1-2 generations after field collection (a), 5-6 generations (b) and 13-14 generations (c). The sample size is 10-16 individuals in each location and treatment

Population structure and differentiation

The eleven microsatellites used for the population genetic analysis showed substantial polymorphism, allele numbers ranging from 10 (Nv303) to 27 (Nv319) (Table 2.2). Single-locus F_{ST} values (Weir & Cockerham, 1984) ranged from 0.025 (Nv205) to 0.152 (Nv322) with an average F_{ST} over all loci of 0.069 (95% confidence intervals: 0.049, 0.098 after bootstrapping over loci) (Table 2.2). Pairwise comparisons of F_{ST} values between locations revealed an appreciable level of genetic differentiation between populations (Table 2.3). The extreme southern and northern populations (COR, OUL) and the population from Latvia (LAT) are significantly different from all other populations, except for the comparison SWI-OUL.

The isolation by distance analysis (Mantel test for matrix correlation) showed a slightly significant correlation between pairwise F_{ST} and geographic distance (R^2 = 0.153, P = 0.035), that is mainly caused by the two extreme populations COR and OUL. When excluding either the COR or the OUL population, the correlation is not significant (data without COR: R^2 = 0.01, P = 0.36, data without OUL: R^2 = 0.08, P = 0.18).

Table 2.2: Population genetic data of the seven sampling locations. Shown are the number of alleles sampled, the expected heterozygosity (H_T; gene diversity) and single locus F_{ST} values for each of 11 microsatellite markers. The bottom line averages values over all markers. Standard errors (SE) were obtained after jackknifing over populations or loci. *n* = sample size (individuals genotyped)

Marker	No. alleles	H_T COR (*n*=18)	H_T SWI (*n*=18)	H_T SCH (*n*=15)	H_T HAM (*n*=17)	H_T LAT (*n*=20)	H_T TUR (*n*=22)	H_T OUL (*n*=26)	F_{ST}±SE
Nv26	18	0.455	0.885	0.783	0.888	0.833	0.918	0.779	0.093±0.052
Nv107	23	0.894	0.91	0.896	0.89	0.936	0.916	0.743	0.041±0.026
Nv118	16	0.898	0.905	0.814	0.852	0.917	0.857	0.797	0.062±0.017
Nv200	18	0.858	0.89	0.86	0.912	0.882	0.923	0.696	0.061±0.035
Nv205	22	0.946	0.902	0.913	0.933	0.872	0.913	0.792	0.025±0.021
Nv229	25	0.857	0.909	0.807	0.91	0.896	0.945	0.825	0.072±0.024
Nv301	19	0.936	0.932	0.859	0.841	0.79	0.94	0.74	0.059±0.028
Nv303	10	0.625	0.773	0.58	0.5	0.561	0.687	0.742	0.113±0.055
Nv319	27	0.833	0.905	0.792	0.917	0.898	0.945	0.869	0.076±0.019
Nv320	19	0.9	0.897	0.868	0.877	0.837	0.832	0.887	0.029±0.009
Nv322	12	0.512	0.801	0.814	0.728	0.721	0.818	0.688	0.152±0.079
Average	19	0.792	0.883	0.817	0.841	0.831	0.881	0.778	0.069±0.011 over loci

Table 2.3: Pairwise F_{ST} values between European populations for all loci combined

	COR	SWI	SCH	HAM	LAT	TUR	OUL
COR	-	0.0821*	0.1022*	0.1074*	0.1089*	0.089*	0.1541*
SWI		-	0.0135	0.0141	0.0369*	0.0093	0.0438
SCH			-	0.0339	0.0873*	0.0416	0.0889*
HAM				-	0.0665*	0.0389*	0.0645*
LAT					-	0.0388*	0.0856*
TUR						-	0.0743*
OUL							-

*Significant values at the nominal level of 5% after Bonferroni correction following G-statistics (as implemented in F-stat)

DISCUSSION

This study shows a latitudinal cline in three aspects of maternal photoperiodic diapause induction for field lines of *Nasonia vitripennis*: the proportion of diapausing offspring, the switch point (photoperiodic counter) and the critical photoperiod (an expression of the photoperiodic timer). The observed variation indicates that photoperiod is an important environmental factor for diapause induction. The fact that the populations from different latitudes responded differently to the applied LD regimes indicates an important genetic component for diapause induction. Therefore, we conclude that maternal diapause induction in *Nasonia vitripennis* is an evolutionary adaptive response to a periodically fluctuating environment for which photoperiod is one of the key cues.

In our study, we found a positive correlation between latitude and proportion of diapausing individuals. The observed pattern is likely due to differential adaptation to local environments as a result of natural

selection. In northern regions, winters are characterized by severe climatic conditions that do not allow normal development and reproduction, while in southern regions winters are mild and might allow survival without diapause. Thus, selection for diapause response in the North is expected to be stronger than in the South resulting in a higher proportion of diapausing individuals. A similar latitudinal cline has been previously described in other species (Schmidt *et al.*, 2005; Scharf *et al.*, 2010; Leisnham *et al.*, 2011). For example, in *Drosophila melanogaster*, the incidence of adult diapause was positively correlated with latitude in populations in Eastern North America (Schmidt *et al.*, 2005).

We also found a positive correlation between latitude and critical photoperiod. Together with the results of latitudinal variation in the proportion of diapausing individuals discussed above, this cline in critical photoperiod provides strong indications for adaptive evolution of diapause in *Nasonia vitripennis* and stresses the importance of photoperiod as the main cue for approaching seasonal change. As northern winters arrive earlier in the year compared to southern ones, populations at high latitudes have to be able to respond swifter to longer photoperiod than southern ones for a timely induction of diapause. The same type of cline was observed for natural populations of the pitcher plant mosquito *Wyeomyia smithii*, for which critical photoperiod also varied along a latitudinal gradient in North America (Bradshaw & Lounibos, 1977). Similar results were obtained in species with diapause at different developmental stages like *Drosophila montana* which possesses an adult reproductive diapause (Tyukmaeva *et al*., 2011) and the butterfly *Sericinus montelus* which has a pupal diapause (Wang *et al.,* 2011). The

estimated critical photoperiod could be used as an indication for the time of the year during which populations start producing diapause offspring. In our case, the long critical photoperiod estimated for OUL occurs approximately on August 11th, whereas the short critical photoperiod of COR corresponds to October 24th. This reflects a difference in timing of diapause induction according to local seasonality. However, different environmental factors play a role in diapause response in nature and may modify the date at which it is beneficial to start producing diapause offspring.

A central aspect of photoperiodic diapause response in *N. vitripennis* is the switch point, measured as the maternal age at which the adult female switches to the production of diapausing offspring. The switch point corresponds to the number of inductive LD cycles accumulated during the sensitive stage necessary for inducing the diapause response and it varies according to latitude and photoperiod. The early switch of northern populations in short photoperiods might represent an evolutionary adaptation to the very rapid seasonal change characteristic of high latitudes. Individuals in these environments respond fast enough to photoperiodic change in order to be able to produce diapausing offspring that will survive winter conditions. Accordingly, southern females inhabit environments characterized by a gradual seasonal change and have a late switch point which allows the production of normal developing offspring during the major part of adult life. As the environmental conditions are generally favourable for a long period, the chance that the offspring will survive and reproduce within the same season is high. Field experiments on induction of diapause could provide

information on the voltinism of *N. vitripennis* in different localities, necessary for understanding the importance of latitudinal variation in switch point.

Photoperiodic diapause response in *N. vitripennis* is a threshold trait whose phenotypic expression is discontinuous and the two phenotypic classes can easily be recognized: in every oviposition event, diapausing and non-diapausing broods can be clearly distinguished and mixed broods are rare, indicating a fast physiological change. The adult female is sensitive to photoperiodic cycles and accumulates the stimuli every day until a specific threshold level is reached and the physiological cascade that leads to the diapause response is activated. The threshold level that activates diapause response depends on the type of stimuli and on the origin of the line. We could rule out an effect of senescence on variation in switch point, as described by Reznik *et al*. (2002) and Yang *et al*. (2007), by showing that life span does not vary under different photoperiodic conditions. In addition, the switch to diapause induction represents the manner in which day length expresses its effect on diapause production. Nonetheless, the strong effect of photoperiod could mask a possible effect of aging which (if present) could be investigated under constant light in populations from different latitudes.

The described variation in switch point indicates that in *Nasonia*, clinal adaptation applies to a cue perceived during the sensitive stage (the mother) that is being effectuated in the next generation. In evolutionary terms, natural selection operates on the threshold number of photoperiodic cycles required to elicit the diapause response and generates the observed latitudinal clines as a consequence of the

environmental cycles and photoperiods that characterise each latitude.

Our results are consistent with those of Saunders who identified photoperiod as the main diapause-inducing factor in laboratory lines of *Nasonia* (Saunders, 1966a). In his pioneering studies, Saunders also observed a difference in diapause response in two lab strains originated from two geographic locations. However, his studies on diapause induction in *N. vitripennis* did not consider natural variation in diapause response. Therefore, our work complements the studies of Saunders, by demonstrating for the first time the importance of adaptive photoperiodic response in diapause induction in *N. vitripennis* in nature. We further showed that natural selection generates the clinal variation through its action on the threshold level of LD cycles required for diapause induction. Following the proposal of Saunders (2002) to divide photoperiodism into two components, timer and counter, we observed that both components show a latitudinal cline by themselves and are correlated. Interestingly, the relationship between timer and counter was consistent over the different latitudes indicating that the photoperiodic machinery becomes increasingly sensitive to short days (or long nights) towards high latitudes. Short photoperiods, measured by the counter, trigger an earlier switch point in northern populations compared to southern ones. This shows the interrelationship of the timer-counter system and may indicate an overlapping genetic architecture, perhaps involving the circadian clock genes.

Interestingly, we neither found a line completely lacking the ability to undergo diapause nor one that entered into obligate diapause at every generation regardless of environmental conditions. This observation

confirms that the difference in diapause response between the lines is based on variation in sensitivity to photoperiod, and not a developmentally fixed pattern to undergo diapause *per se*. However, it cannot be excluded that strains exist which do not possess a photoperiodic diapause, for example at latitudes lower than our southernmost location where photoperiod may not represent a good cue for seasonal change. Moreover, some strains with obligate diapause could be present at very high latitudes outside the studied range, where extremely short summers and rapid seasonal change do not allow more than one generation per year. During our fieldwork, we were able to collect a single individual in a location in very northern latitude (Finland, Kilpisjärvi, 69° 2'39.44"N, 20°48'11.88"E). This female was used to establish an isofemale line but only produced diapausing larvae and could not be included in our experiments. This shows that *N. vitripennis* has extended its geographical range to extreme latitudes making it a cosmopolitan species which has adapted to many different climatic conditions. *N. vitripennis* represents therefore a powerful model to investigate the genetic basis of variation in diapause and other adaptive traits.

Population differentiation analysis using pairwise F_{ST} comparisons based on microsatellite markers, showed an appreciable level of genetic differentiation between some of the tested populations. Previous studies indicated that gene flow between *Nasonia vitripennis* populations in North America is high within a range of about 100 km and limited between populations that are separated by 300 km or larger distances, particularly if large bodies of water or mountain ranges are present between the

locations (Grillenberger *et al.*, 2009). In our study, the F_{ST} values measured between distant locations are similar to the ones previously reported for North American populations confirming that gene flow is restricted. Studies in other insect species demonstrated that local adaptation can lead to clinal variation in different phenotypic traits despite a high level of gene flow measured with neutral genetic markers (Demont *et al*., 2008; Sarup *et al*., 2009; Tyukmaeva *et al*., 2011). Comparisons of populations at similar geographical distances show that our measured F_{ST} values are generally larger than those of the dung fly *Scatophaga stercoraria* and the northern malt fly *Drosophila montana*. For example, the F_{ST} value for yellow dung fly populations from Finland and Germany (about 1500 km) was 0.011 (Demont *et al*., 2008), whereas the value for our OUL and HAM populations, at similar distance, was 0.064. Similarly, F_{ST} values between populations of *Drosophila montana* in Finland were negligible in all pairwise comparisons, including the most southern and northern populations (845 km) that have F_{ST} value of 0.006 (Tyukmaeva *et al*., 2011), whereas in our case the F_{ST} value between TUR and OUL (450km) was 0.07. The difference between flies and *Nasonia* is likely caused by the subdivided population structure of *Nasonia* due to a patchy distribution of its host (Grillenberger *et al*., 2008) and the inability of the flightless males to disperse.

The measured genetic differentiation does not correlate entirely with the latitudinal cline for diapause response and the overall isolation by distance effect was mainly due to the two most distant populations. Hence, the observed geographic variation in diapause appears to be in a selection – migration balance rather than due to neutral processes (e.g.

genetic drift) (Leinonen *et al*., 2008). When more information is available about the genetic basis of diapause in *Nasonia vitripennis*, population genomic studies may reveal signatures of selection in the genes involved. The reported natural variation in photoperiodic diapause combined with the genomic tools available for this species offers an excellent opportunity to investigate the genetic basis of diapause and its adaptive variation.

In conclusion, the present work represents the first study on variation of photoperiodic diapause induction in *Nasonia vitripennis* in relation to natural seasonal cycles in different geographical locations and provides indications for adaptive evolution of this trait. The importance of the switch point for diapause induction in the establishment of the latitudinal cline indicates that future studies aiming to identify the genetic basis of diapause variation should focus on the sensitive maternal stage, on the perception of photoperiodic cues and on processing of the information derived from these cues.

ACKNOWLEDGEMENTS

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Chapter 3

The quantitative genetic basis of photoperiodic diapause induction in *Nasonia vitripennis*: modes of inheritance and QTL study

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ABSTRACT

Diapause is a life history trait used by many insect species to survive adverse environmental conditions. Despite the large number of studies showing phenotypic variation in diapause response as a result of local adaptation, little is known about the genetic basis of this important adaptive trait. In this chapter we examine the genetic basis of photoperiodic diapause induction in the parasitic wasp *Nasonia vitripennis* which has a larval diapause induced by the mother. We use two field strains originated from a northern (Oulu, Finland) and a southern (Corsica, France) location which show variation in maternal sensitivity to photoperiod. Reciprocal crosses between the two lines demonstrate that variation in photoperiodic diapause response is genetically determined and mainly depends on nuclear genes with no cytoplasmic effects. A combination of QTL analysis and candidate gene approach revealed two large genomic regions involved in diapause variation, located on chromosome 1 and chromosome 5. Interestingly, the highest QTL peak corresponds to the locus of the two candidate genes *period* and *cycle* suggesting a link between clock genes and adaptive photoperiodic response.

INTRODUCTION

Life in seasonal environments requires coordination with fluctuating annual cycles of light, temperature and food availability. Consequently, the ability to use environmental cues to reliably anticipate seasonal changes is subject to natural selection. Many insect species survive adverse environmental conditions by entering in a state of physiological dormancy called diapause (Tauber *et al.,* 1986; Danks, 1987). Diapause induction, therefore, can be considered an adaptive trait, strongly moulded by seasonality. Studies reporting latitudinal variation in diapause response confirm the adaptive value of this trait and its variation according to the prevailing local environmental seasonal cycle (reviewed in Hut *et al*., 2013). Most insects use photoperiod as a reliable cue to anticipate seasonal changes and as a stimulus for entering diapause. Previous studies revealed that photoperiodic diapause induction is under genetic control, albeit with different modes of inheritance. For example, simple Mendelian inheritance with diapause dominant over non-diapause phenotype is found in the yellow dung fly *Scatophaga stercoraria* (Demont & Blanckenhorn, 2008) and polygenic control in the pitcher plant mosquito *Wyeomyia smithii* (Mathias *et al*., 2007). However, the precise genetic architecture underlying photoperiodic diapause induction and the loci responsible for its variation remain essentially unknown.

More than 70 years ago (Bünning, 1936) it was hypothesized that photoperiodism and the internal circadian clock are causally linked and are controlled by the same underlying mechanisms. This hypothesis is based on the fact that both circadian clock machinery and photoperiodic

diapause are regulated by natural light:dark cycles and might therefore be under control of the same genes. Ever since, numerous studies have attempted to find empirical support for this view. While the circadian clock machinery of insect model species such as *Drosophila melanogaster* is well known and characterized (Hardin, 2005), the molecular and genetic basis of seasonal photoperiodic response remain poorly understood. In *D. melanogaster* the functional mechanism of the circadian clock is based on a series of feedback loops in which different clock components are expressed in a rhythmic manner, regulated by the external photoperiodic cycle. Light acts as trigger and entrainment cue for the rhythmic expression of specific genes, named "clock genes", whose specific role in the *Drosophila* circadian clock is well described (Hardin, 2005). These key genes are highly conserved across different species and represent good candidates for the genetic basis of insect photoperiodism and its adaptive variation (Meuti & Denlinger, 2013).

We have shown earlier that in the parasitoid *Nasonia vitripennis*, photoperiodic induction of diapause is an adaptive trait that varies geographically over a latitudinal cline (Paolucci *et al*., 2013; chapter 2, this thesis). In this species, diapause occurs at the larval stage and is induced by the adult mother (sensitive stage). Hence, the mother is able to perceive the environmental photoperiodic cues and translates these cues into information that leads to different developmental programmes of their offspring: diapause or non-diapause (Saunders, 1965). Depending on the photoperiodic conditions, adult females produce normal developing offspring early in life and switch to the production of diapausing offspring after a critical threshold number of photoperiodic cycles. This number of

cycles is defined as the *switch point* and is the key aspect of adaptive diapause in *N. vitripennis.* The clinal variation of diapause induction is expressed as variation in this aspect, with early switch points in northern populations and late switch points in southern populations. Such a clear photoperiodic response and the possibility to isolate the maternal sensitive stage subject to selection, make *Nasonia* a very good system for investigating the genetic basis of adaptive diapause response.

In this chapter, we present the first analysis of the genetic basis of photoperiodic diapause variation in *Nasonia vitripennis*. Reciprocal crosses between two isofemale lines, originating from a northern (Oulu, Finland) and a southern (Corsica, France) location, were used to investigate the patterns of diapause inheritance and to identify loci involved in switch point variation by a combination of QTL analysis and candidate gene approach which focused on clock genes.

MATERIALS AND METHODS

Experimental lines

Two *Nasonia vitripennis* isofemale lines established from strains originating from Oulu, Finland and Corsica, France (Paolucci *et al*., 2013) were used for the genetic cross experiments and QTL analysis. These lines are named “northern line” and “southern line”, respectively. The lines were maintained on *Calliphora spp*. pupae as hosts in mass culture vials under non-diapause conditions (light:dark cycle 18:6, 20–25 °C).

Cross design

Virgin females from the northern line (genotype: NN) and the southern line (genotype: SS) were collected as pupae from the hosts and placed in cotton-plugged 60 mm x 10 mm polystyrene tubes together with male pupae from the same line or the other line (haploid male genotype: N or S), resulting in four types of crosses: two intra-line crosses NxNN and SxSS, and two reciprocal inter-line crosses, NxSS and SxNN. Since sex ratio in *Nasonia* is highly female biased, four females were placed with one male in each tube. In total, 40 females and 10 males were used for the intra-line crosses and 120 females and 30 males for the inter-line crosses. After eclosion and mating, 3 hosts were provided to the females for oviposition. These hosts were subsequently kept at 20°C and continuous light until adult parasitoids emerged after 3 weeks. These F1 individuals were allowed to mate for one day and females were subsequently isolated in tubes with two hosts and placed under diapause inducing photoperiod (light:dark 14:10, 20°C). Diapause response was scored for 30 F1 females from both intra-line cross (NN and SS females) and 100 females from the inter-line crosses (NS and SN females). F1 females from reciprocal crosses have 50% northern genome and 50% southern genome, but differ for the maternally inherited cytoplasm, that is either S or N (denoted as “NS[S]” and “SN[N]”) (Fig. 3.1A).

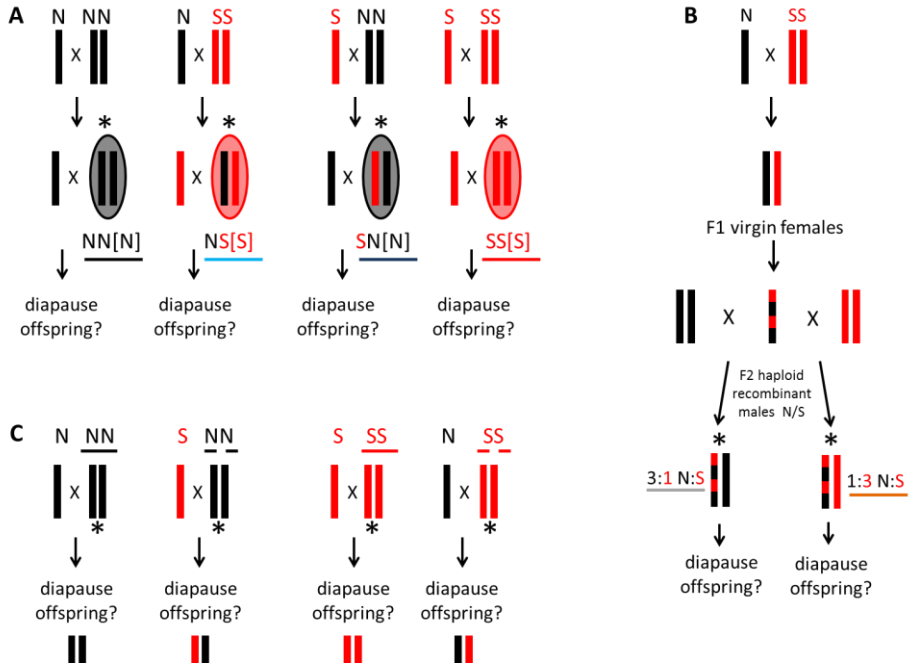

Figure 3.1: Scheme of the cross design. The switch point for diapause induction is measured in females marked with stars. The colour of the lines underneath the female symbol is the same as used in Figure 3.2 that shows the phenotypic response. The switch point is determined by scoring diapausing larvae in their offspring. **A)** Intra-line crosses generate F1 homozygous NN and SS females (outer crosses). Reciprocal inter-line crosses generate F1 heterozygous NS[S] and SN[N] females with different maternally inherited cytoplasm (inner crosses). Oval colours indicate cytoplasm, grey is from northern and red from southern line. **B)** Simplified cross design used to generate the mapping population of recombinant individuals. For clarity, only one parental cross is shown. Inter-line crosses generated F1 heterozygous females which produced haploid recombinant males used for genotyping. Each F2 male was backcrossed with females from the northern and southern line to generate F3 females with 3:1 and 1:3 north:south genome proportions. **C)** Switch point is measured in NN and SS females mated with males of their own line or the other line. Females of the same genotype (NN or SS) produced offspring with different genotype depending on the male line.

To establish the mapping population, two males and two females from both the northern and the southern line were isolated as pupae from the mass culture and placed as pairs in vials in four combinations (NxNN, SxSS, NxSS, SxNN). After eclosion and mating, hosts were provided to each of the four females and after egg laying, they were kept at standard maintenance conditions. The eight parental individuals were stored in 70% ethanol at -20 °C for future analysis of the genotype. F1 individuals from the pure line crosses were intercrossed to produce the F2 generation which will be available for subsequent backcrosses. In parallel, F1 females from inter-line crosses were isolated as virgins to produce F2 recombinant haploid males, noted as N/S. Four hundred recombinant males were individually backcrossed to a F2 virgin female from both the southern and northern line. After mating, the recombinant males were stored in 70% ethanol at -20 °C for future genotyping. Offspring from these backcrosses developed at 20°C and continuous light. Eight hundred F3 female offspring from these backcrosses were individually scored for photoperiodic diapause response upon exposure to light:dark cycle 14:10 (see below for diapause phenotyping), with equal numbers from both backcrosses (Fig. 3.1B).

In order to investigate the effect of offspring genome on photoperiodic diapause induction and potential interactions between southern and northern genome, an additional cross experiment was performed. Thirty females from each isofemale line were mated with males of the other line and their diapause response was compared to that of 30 females from each line mated with males of their own line. The resulting four combinations were: NxNN, SxNN, SxSS, NxSS. In this

comparison, females from the same genotype generate offspring with different genotypes depending on the male with which they are mated (e.g. NN females produce always male N offspring but female NN offspring when mated with N males and female SN offspring when mated with S males). Since diapause response is induced by the mother but is manifested and scored in the offspring, such comparison allows to test the potential effect of offspring genotype on photoperiodic diapause (Fig. 3.1C)

Diapause phenotyping

The general methodology for measuring the photoperiodic diapause response in *N. vitripennis* is described in chapter 2 of this thesis. Briefly, single mated females were placed in a vial with two hosts under light:dark cycle 14:10, 20°C. Females were exposed to the treatment for the first 20 days of their adult life and provided with two fresh hosts every other day. The parasitized hosts were kept at 20°C and constant light. Diapause response was determined by opening the hosts 21 days after egg laying and scoring for presence of diapausing larvae. As mixed broods, containing a mix of diapausing and non-diapausing offspring, are very rare, diapause can be considered a binary trait, so each set of two hosts was scored as either “diapause” or “no diapause”. For each female, the diapause response was expressed as *switch point* corresponding to the maternal age (in days) at which she switches from producing non-diapausing to diapausing offspring. The switch point represents the threshold number of LD cycles required to trigger the diapause response. The statistical analysis was performed using the R statistical software (R Development Core Team

2012). Survival analysis was used to analyse the switch point for diapause induction. All phenotypic data were analysed using Cox proportional hazard models (package *survival* in the R language).

Microsatellite genotyping and candidate genes

Genomic DNA was extracted using a standard high salt-chloroform protocol (Maniatis *et al.*, 1982). Thirty five microsatellite markers were amplified using the Qiagen multiplex PCR kit according to manufacturer's recommendations (PCR profile: 15 min at 94 °C, followed by 30 cycles of 30 sec at 94 °C, 1.5 min at 57 °C and 1 min at 72 °C, followed by 45 min at 72 °C)(appendix 1.1). The length of the amplified fragments was determined using the Applied Biosystems 3730 DNA Analyzer and analysed using GENE MAPPER v4.0 (Applied Biosystems, Carlsbad, CA, USA). From this set, twenty three diagnostic markers were selected to analyse the F2 recombinant males (appendix 1.2).

Diagnostic SNP markers were identified in three candidate genes located in intron 8 of *period* (NasoniaBase NV16428-RA), intron 3 of *cycle* (NasoniaBase reference NV13263-RA) and intron 7 of *cryptochrome* (NasoniaBase reference NV13040-RA) (appendix 2). These markers were used to genotype a subset of F2 recombinant males (98 individuals for *period*, 62 for *cycle*, 71 for *cryptochrome*) (appendix 3: sequencing protocol).

The molecular markers did not deviate from a 1:1 segregation (following Bonferroni correction, $P > 0.05$). The intra-specific linkage map was constructed based on the genotypes of 400 recombinant males using the package *R/qtl* in the R language. Five linkage groups were found,

corresponding to five chromosomes of *Nasonia* and the order of markers in the linkage groups conform to the previously described linkage maps for *Nasonia* (Beukeboom *et al.*, 2010; Pannebakker *et al.*, 2010; Koevoets *et al.*, 2012).

QTL analysis

For each F2 recombinant male, the probability of the allelic state at every cM map position, conditional to the observed genotype for the segregating markers, was estimated using a hidden Markov model, allowing for genotyping errors and missing genotype data, as implemented in the R package *R/qtl* (Broman & Sen, 2009). The QTLs were mapped to the genome using Haley-Knott regressions, involving individual phenotypes of 800 females from both backcrosses regressed on the conditional genotype probability of their F2 sire to carry the northern allele at every cM. In the regression model, the genotype probabilities multiplied by the additive and dominant coefficients were included as explanatory variables. A position with minimal residual variance was considered to be a putative QTL position. The residual variance of the full model RSS_1, including additive and dominance effects, was used to infer LOD scores, using the formula $LOD=n/2 \log_{10} (RSS_0/RSS_1)$ where n is the sample size and RSS_0 is the variance of the null model (Broman & Sen, 2009). The genome-wide significance threshold was estimated by performing 1000 Churchill-Doerge permutations and taking the 5% cut-off as significant threshold value (Churchill & Doerge, 1994).

The percentage of phenotypic variance explained by the detected QTLs was determined by building a regression model in which additive and

dominant terms of both significant QTLs and all possible interactions were tested as explanatory variables. A Chi-squared test for likelihood was used to select the best QTL model containing only significant explanatory factors and proportion of phenotypic variance explained by the identified QTLs was assessed with the formula $VAR=1- RSS_{QTL}/RSS_0$ where RSS_{QTL} is the residual variance of the best QTL model and RSS_0 is the residual variance of the null model without genetic terms (Pannebakker *et al.*, 2011). The QTL analysis was performed in two steps. In the first analysis, 23 microsatellite markers were used. Subsequently, the three SNP markers in the candidate genes were added and a new QTL analysis was performed**.**

RESULTS

Inheritance of photoperiodic diapause induction

Adult *N. vitripennis* females from northern and southern lines showed a significantly different diapause switch point upon exposure to a 14:10 circadian light:dark cycle. Switch points were 4.22±0.36 days for the northern line and 12.21±0.54 days for the southern line (Fig. 3.2A). Reciprocal crosses between the northern and the southern lines yielded F1 females with average diapause switch points of 8.82±0.21 days and 9.04±0.18 days for SN[N] and NS[S] females, respectively (Fig. 3.2A). These switch points were significantly different from either parental line but not from each other (*post hoc* multiple comparison for survival analysis, $P < 0.05$). Since F1 females from the reciprocal crosses show similar switch points (*post hoc* multiple comparison for survival analysis, $P = 0.98$), it can

be concluded that cytoplasmic effects are not responsible for the variation in diapause induction.

Virgin F1 NS and SN females produced F2 haploid males with a recombinant N and S genome of 50% N and 50% S. These males were backcrossed with females from both the northern and southern lines to yield F3 diploid females with half N/S recombinant genome (from the recombinant father) and half original N or S genome (from the mother), representing proportions of N:S genomes of 3:1 and 1:3, respectively. The mean switch point was 6.57±0.08 days for 3:1 N:S females, and 10.19±0.13 days for 1:3 N:S females. These values were both significantly different from each other and from all other genotypes tested (Fig. 3.2A, *post hoc* multiple comparison for survival analysis, $P < 0.05$). Notably, there is a significant correlation between the genomic composition in terms of relative proportion of northern and southern genomes (1:0 northern to southern genome in NN females and vice versa in SS females, 3:1 northern to southern genome in northern backcrossed 3:1 N:S females and vice versa in 1:3 N:S females and 1:1 in NS and SN females) and the time of switch point ($Z = 22.16$, Kendall's rank correlation tau = 0.60, $P < 0.01$). These results indicate that maternal photoperiodic diapause induction is a genetically determined trait with no cytoplasmic effects influencing its variation.

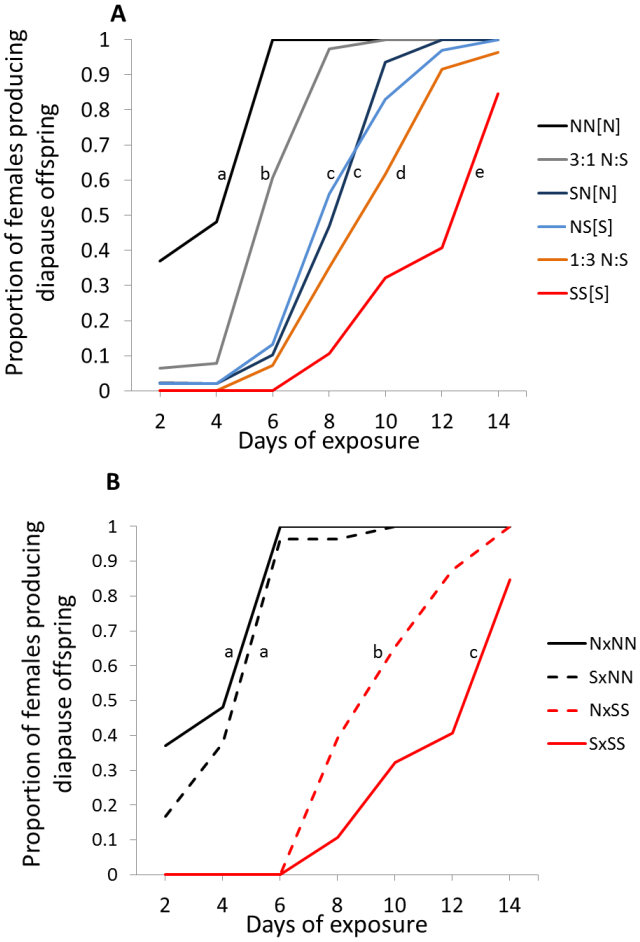

Figure 3.2 Diapause response of *Nasonia vitripennis* females under light:dark cycle 14:10. **A)** Effect of female genotype. Lines represent the responses of F1 females from pure crosses (NN[N] and SS[S]), inter-line crosses with different cytoplasm (NS[N] and SN[S]) and F3 backcross females (3:1 N:S for the backcross to the North and 1:3 for the backcross to the South). Pure lines have 100% of N or S genome respectively. F1 Females originated from inter-line crosses have 50% of S and N genome. Backcross females have 75% of N or S genome and 25% of S or N genome respectively. Letters indicate significant difference (post-hoc multiple comparison for survival analysis, $P < 0.05$). **B)** Effect of male genotype and offspring genome. Lines represent the responses of NN females mated with N or S males and SS females mated with S or N males. Letters indicate significant difference (post-hoc multiple comparison for survival analysis, $P < 0.05$)

In an additional experiment, the effect of offspring genomic composition on diapause variation was investigated. The diapause response of NN females mated with S males did not differ significantly from the response of NN females mated with N males (cox proportional hazard model, χ^2 = 1.14, P = 0.28). However, SS females mated with N males showed an earlier switch point (10.16±0.42) compared to SS females mated with S males (12.54±0.54) (cox proportional hazard model, χ^2 = 10.8, P = 0.001). In addition, NN females mated with S males and SS females mated with N males produce offspring with similar nuclear genome (50% northern and 50% southern) but their switch point was significantly different (*post hoc* multiple comparison, P < 0.01) (Fig. 3.2B). Overall, these results suggest that maternal induction of larval diapause is predominantly controlled by maternal genes but can be partially affected by the interaction between the maternal genome and offspring genome, as shown for SS females mated with two types of males having different diapause induction in their offspring.

QTL analysis

The QTL analysis for switch point under the chosen photoperiodic regime revealed two significant QTLs: one located on chromosome 1 at 61 cM ($F_{2,699}$ = 35.13, P < 0.001; 1.5LOD interval 49-77 cM) and one on chromosome 5 at 69 cM ($F_{2,699}$ = 14.21, P < 0.001; 1.5LOD interval 33-95 cM) (Table 3.1, Fig. 3.3). The QTL on chromosome 1 corresponds to the map position of the *period* and *cycle* gene. Although these two genes are separated by approximately 5.8 Mb (Niehuis *et al.*, 2010), no recombination events between the SNPs in *period* and *cycle* were

detected. Local recombination rate in marker clusters in this genomic region near the centromere ranges between 0.077 and 0.518 cM/Mb, perhaps a larger sample size is required to detect recombination between the *period* and *cycle* genes.

Table 3.1: Genome-wide significant QTLs for photoperiodic induction of diapause (switch point) in *Nasonia vitripennis*. The normalized QTL effect is the change in phenotype between homozygous and heterozygous individuals relative to the mean in the northern and southern background. The QTL effect is expressed as additive (a) and dominant (d) effects.

Chromosome	Location (cM)	Variance explained	Background	Normalized QTL effect	a	d
1	61 (*per/cycle*)	9.1%	North South	8.1%. 14.3%	-1.51	-0.79
5	69	3.9%	North South	3.4% 8.5%	-1.43	-0.81

Individual phenotypic values were regressed on the probability of F2 recombinant males to carry the northern allele (see materials and methods) and as a consequence negative values of the QTL additive effects, that were observed for both QTLs, indicate an association of the northern allele with an earlier switch point in both backgrounds (fast diapause response). A partial dominance of the northern allele over the southern allele was assessed (degree of dominance for the QTL on chromosome 1 was d/a=0.52 and for the QTL on chromosome 5 was d/a=0.56). As a consequence, the relative change in switch point associated with the QTLs was lower in the northern background than in the southern background (Table 3.1, normalized QTL effects) meaning that the difference in switch point between SN and NN individuals (at the QTL

locus) in northern background was smaller than the difference between NS and SS individuals in the southern background. Overall, the percentage of phenotypic variance explained by both QTLs in an additive manner was 13.7% (QTL linear model, $F_{4,697} = 27.71$, $P < 0.001$). There was no significant effect of interaction between the two QTLs

The candidate gene *cryptochrome* (*cry*) is located on chromosome 5, but fell outside the 1.5LOD interval for the identified QTL on this chromosome, and was therefore not associated with variation in photoperiodic induction of diapause.

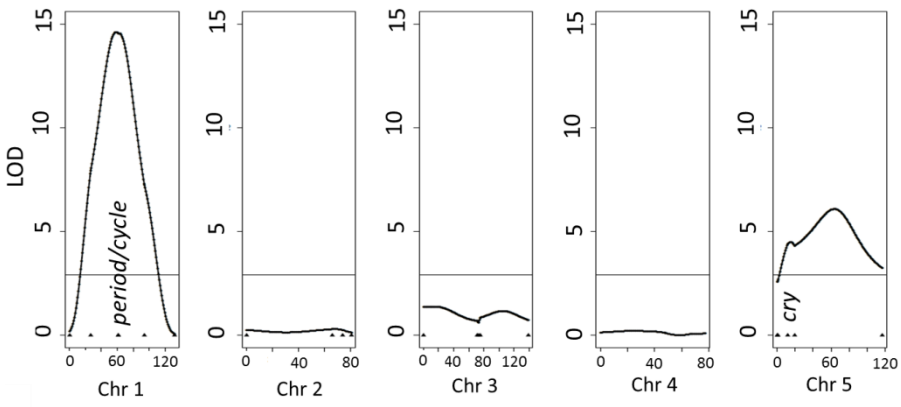

Figure 3.3: Locations of QTLs for photoperiodic diapause induction (switch point) in *Nasonia vitripennis*. The horizontal line corresponds to the 5% genome-wide significance threshold from permutation tests.

DISCUSSION

This work represents the first analysis of the genetic basis of adaptive photoperiodic diapause in the parasitoid wasp *Nasonia vitripennis.* Using genetic crosses between individuals from northern and southern lines, we showed that photoperiodic diapause induction in *N. vitripennis* has a strong genetic component. Through QTL analysis, we identified two genomic loci involved in the variation of the trait. Furthermore, the highest QTL peak of the first chromosome corresponds to the *period/cycle* locus suggesting a role for clock genes in photoperiodism.

Given that larval diapause in *N. vitripennis* is under control of the adult mother, maternal cytoplasmic effects may be expected to influence diapause. However, we did not find significant differences in switch point between reciprocal inter-line crosses, therefore we conclude that the primary source of the between-line variation in diapause response is based on nuclear maternal genes. This result is confirmed by the significant correlation between relative proportion of northern and southern genome in the mother and the switch point. Nevertheless, our second genetic cross showed a partial effect of offspring genome on photoperiodic response. Southern females mated with northern or southern males had offspring with different genotypes (SS offspring when SS mothers are mated with S males and NS offspring when SS mothers are mated with N males) and showed different switch points. Such effect could be explained in terms of epistatic interactions between maternally provided gene products and genes expressed in offspring in the expression of diapause. In fact, we consider the switch point as a female maternal

trait but we measure it in the offspring. In larval diapause of maternal origin, the photoperiodic cues detected by adult female are translated into physiological and/or hormonal factors that affect the developmental pathway of the offspring. It is possible that offspring physiological response to maternal factors is also under genetic control and co-evolved with maternal diapause induction (threshold sensitivity to cues). Therefore a mismatch between maternal and offspring genome, due to different paternal genotype, could disrupt this combination and lead to a different response. Alternatively, the different switch point of SS females mated with S or N males could be due to the effect of seminal fluid proteins provided by the male. These proteins have been studied mainly in *Drosophila* and they are known to induce various types of physiological postmating changes in the females (e.g. they affect oviposition rate) which could in turn have an effect on the induction of diapause in the offspring (Avila *et al.*, 2011). Differently from southern females, northern females have similar switch points, regardless of the genotype of the mating male and thus the offspring. This could be explained by the partial dominance of northern alleles over southern alleles as revealed by the QTL analysis. The dominant northern alleles might mask the effect of the southern alleles present in the offspring genome. Parental effects on diapause induction have been observed in many species (reviewed by Mousseau & Dingle, 1991). For example, crosses between different geographic strains of the blow fly *Calliphora vicina* showed that the larval diapause incidence is entirely controlled by the maternal genetic background without any effect of paternal genome (Mcwatters & Saunders, 1996). In other species, such as the Asian corn borer *Ostrinia furnacalis* (Huang *et al.*, 2013) and the

cotton bollworm *Helicoverpa armigera* (Chen *et al.*, 2012), the induction of diapause is highly influenced by the paternal genome. Thus, the parental effects can be biased towards either parent depending on the species, in line with the high diversity of diapause expression and modes of inheritance. Further investigation of different parental and offspring effects on diapause induction in N. vitripennis are necessary to elucidate possible genotype x environment interactions, genetic compatibility effects and the exact role of offspring genome in the expression of diapause.

The results of the genetic crosses confirmed that variation in diapause response between northern and southern lines is based on genetic variation for photoperiodic counter expressed as switch point. Figure 3.2 shows that in the first days of adult life (day 2-6) and later in life (day 14) the proportion of females producing diapause offspring does not differ between pure lines, F1 and backcrossed females. The largest phenotypic variation among female genotypes is observed between day 6 and day 12, confirming that natural selection acts on the maternal sensitivity to environmental cues resulting in different levels of diapause in the offspring. Thus, our approach for measuring the photoperiodic diapause response, in terms of the switch point, is a good way of determining response variation and gives a direction for future studies of the molecular mechanism of adaptive photoperiodic diapause and maternal effect.

The QTL analysis identified two genomic regions involved in variation of switch point for photoperiodic diapause. The QTL peak with highest LOD score is located on chromosome 1 and accounts for 9.1% of

the phenotypic variation. A second QTL peak was found on chromosome 5 explaining a lower percentage of variation (3.1%). The identified genomic regions involved in photoperiodic response cover large fragments of the two chromosomes and potentially contain numerous genes. This is due to the relatively low resolution of the linkage map resulting from the low number of used markers. To better elucidate the underlying genetic basis of adaptive diapause variation and to restrict the QTL peak to a narrower region containing few candidate genes, a larger number of intraspecific molecular markers need to be developed from genomic sequence data. Nevertheless, the applied combination of QTL analysis and candidate gene approach uncovered a locus for photoperiodic diapause induction containing the clock genes *period* and *cycle.* Their position in chromosome 1 precisely corresponds to the QTL peak. This result suggests a relevant role for clock genes in photoperiodic diapause. The diagnostic SNP markers in *period* and *cycle* segregated as a single marker in the F2 backcrosses and therefore we could not disentangle the relative importance of the two genes based on the QTL analysis. Moreover, other genes positioned under the QTL peak and linked to the *period/cycle* locus, cannot be excluded and could explain a larger proportion of phenotypic variance. Nevertheless, the significant association of diapause with the *period/cycle* locus is suggestive of a link between circadian clock genes and photoperiodic response.

The involvement of clock genes in photoperiodic diapause response has been investigated in various insect species (recently reviewed by Koštál, 2011; Saunders & Bertossa, 2011; Meuti & Denlinger 2013; Goto, 2013) using different methodologies including expression

studies, use of mutants (Koštál & Shimada, 2001) and gene knock down through RNAi techniques (Ikeno *et al.*, 2010). Although the results of these studies show directly or indirectly that clock genes play a role in photoperiodic diapause, it is still not known whether the circadian clock mechanism forms the basis for the seasonal photoperiodic diapause response, or whether the clock genes have pleiotropic effects on both traits (Hut *et al.*, 2013). A recent study on *Drosophila triauraria* showed an association between allelic variation in the genes *timeless* and *cryptochrome,* and the incidence of diapause, but no involvement of *period*, *cycle* and *clock* (Yamada *et al*., 2011). However, the authors were able to associate the clock genes with variation in diapause incidence, but not with variation in the slope of the photoperiodic response. It is still very well possible that *period* and *cycle* are involved in other aspects of photoperiodic response in *D. triauraria*, such as the photoperiodic timer or photoperiodic counter, which constitute the two main components of the photoperiodic clock (Saunders, 2002). In another study, the involvement of the clock gene *timeless* in the critical photoperiod for diapause induction was shown through a QTL analysis using northern and southern strains of the pitcher plant mosquito *W. smithii*. The gene *timeless* did not map directly to a QTL but interacted epistatically with it, suggesting that the role of this gene in photoperiodic timer (expressed as critical photoperiod) is independent from its function in the circadian clock (Mathias *et al.*, 2007).

In this study we reported the association of the locus *period/cycle* with variation of switch point for maternal induction of photoperiodic diapause. This result constitutes the basis for the investigation of the

function of clock genes in maternal threshold sensitivity to environmental photoperiodic cues and it gives a direction to further examine the correlation between clock gene variation and natural variation in photoperiodic response which will in turn shed light onto the genetic basis of adaptive variation of photoperiodic diapause induction.

Chapter 4

A latitudinal cline in *period* gene polymorphism is correlated with photoperiodic diapause response in *Nasonia vitripennis*

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ABSTRACT

Understanding the evolutionary link between the circadian clock and photoperiodism is a major goal of research in chronobiology. The role of clock genes in seasonal photoperiodic response, such as insect diapause, is the subject of a longstanding debate. A useful approach to investigate the involvement of clock genes in diapause is to study their genetic variation in natural populations that show adaptive variation in photoperiodic response. In this chapter we describe the correlation between a genetic polymorphism in the clock gene *period* and variation in photoperiodic diapause induction in the parasitoid wasp *Nasonia vitripennis*. We sequenced exons of *period* in individuals from two field lines originating from a northern (Oulu, Finland) and a southern (Corsica, France) location. We identified 3 haplotypes called per_{N1}, and per_{N2} in the northern line and per_{S} in the southern line, based on non-synonymous SNPs. Laboratory crosses in lines with different genotypes showed that the haplotype per_{N1} was associated with stronger diapause response compared to per_{N2} and per_{S}. Moreover, per_{N1} was dominant over per_{N2} and both northern haplotypes were dominant over per_{S}. We further investigated the polymorphism in *period* in seven *N. vitripennis* populations from a North-South cline and found a latitudinal cline in allele frequency for two *per* alleles, defined by the haplotypes per_{N1} and per_{S}. The clines in allele frequency correlate with the latitudinal cline in switch point for photoperiodic diapause induction previously described for these populations. The results of this chapter confirm the involvement of the clock gene *period* in adaptive variation in photoperiodic diapause

response in *N. vitripennis* and suggest a functional link between the circadian clock and photoperiodism.

INTRODUCTION

One of the most debated topics in evolutionary chronobiology is the hypothesis of an evolutionary link between the circadian clock and seasonal photoperiodism. While many scientists agree on the functional involvement of the circadian system in the photoperiodic response, others consider the two mechanisms physically separated and functionally independent (Koštál, 2011). Despite the increasing number of theoretical and empirical studies in this topic, the debate remains still open and additional research is needed to reach consensus. The molecular basis of the circadian clock mechanism of model species is well known and clock genes represent the best candidates for investigation of the link between circadian clock and photoperiodism. Given the large number of studies showing natural latitudinal variation in photoperiodic response (e.g. insect diapause), it is surprising that only a few have investigated correlated clinal genetic variation at candidate clock genes (Kyriacou *et al.*, 2008). Such studies are very useful to understand the genetic basis of adaptive variation and to evaluate the hypothesis of a shared genetic basis between the circadian clock and seasonal photoperiodic response.

Studies on variation in frequency of *period* and *timeless* alleles in natural populations of *Drosophila melanogaster* are presently the most well-known examples of latitudinal clines in clock gene polymorphism in insects. Screening natural populations in Europe revealed that *period* was present in two main variants which differ in the length of a Thr-Gly repeat coding sequence. The frequencies of the two forms follow opposite latitudinal clines with the variant $(Thr\text{-}Gly)_{20}$ increasing towards northern

latitudes and the variant $(Thr\text{-}Gly)_{17}$ increasing towards southern latitudes (Costa *et al.*, 1992). A similar clinal variation in *period* allelic frequency was observed in natural populations in Australia (Sawyer *et al.*, 2006). Similarly, the frequency of two alleles of *timeless* encoding for a short protein form *S-TIM* and a long form *L-TIM* follows a latitudinal cline in European populations (Tauber *et al.*, 2007). Although the clines in *period* and *timeless* suggest a role for these two genes in the adaptation to different environments, the polymorphisms could not be directly correlated to a latitudinal cline in diapause response in *D. melanogaster* in Europe.

The parasitoid *Nasonia vitripennis* has larval diapause that is induced by the mother upon experiencing short photoperiods. We demonstrated that photoperiodic diapause response in European *Nasonia* populations follows a latitudinal cline, in terms of the age (i.e. number of experienced photoperiodic cycles) and photoperiod at which females switch to producing diapausing eggs. This maternal sensitive stage is expressed as an early switch point in northern populations, and a late switch point in southern populations (Paolucci *et al.,* 2013; chapter 2, this thesis). As described in chapter 3, diapause induction in *N. vitripennis* has a polygenic basis. The switch point is governed by two main genomic regions: one on chromosome 1 and one on chromosome 5. Interestingly, the main identified QTL peak on chromosome 1 corresponds to SNP markers located in the clock genes *period* and *cycle*, suggesting a role of these genes in the observed variation of switch point for photoperiodic diapause induction.

In this chapter we present the analysis of sequence variation in the

period gene and its association with photoperiodic diapause response in *Nasonia vitripennis.* In addition, we document variation in the frequency of *period* haplotypes and the correlation with clinal switch point variation for diapause induction.

MATERIALS AND METHODS

Experimental lines

The source material for this study is a panel of isofemale lines established from field material collected in seven locations in Europe along a North-South latitudinal gradient (OUL (Finland, Oulu): 65°3’40.16”N, 25°31’40.80”E; TUR (Finland, Turku): 61°15’40.53”N, 22°13’23.96”E; LAT (Latvia): 56°51’22.56”N, 25°12’1.38”E; HAM (Germany, Hamburg): 53°36’23.62”N, 10°10’17.74”E; SCH (Germany, Schlüchtern): 50°19’56.10”N, 9°30’47.00”E; SWI (Switzerland): 46°44’9.14”N, 7°6’57.34”E; COR (France, Corsica): 42°22’40.80”N, 8°44’ 52.80”E). The details about collection sites and sampling methodology are given in Chapter 2. Two isofemale lines originating from Oulu, Finland (“northern” line) and Corsica, France (“southern” line) were selected for the first screening of sequence variation in the clock gene *period* and for the crosses in the laboratory. The lines were maintained on *Calliphora spp* pupae as hosts in mass culture vials under diapause-preventing conditions (long photoperiod, 20–25 °C).

Period *haplotypes*

The full DNA sequence of *period* with predicted intron/exon structure was downloaded from NasoniaBase (Reference name NV16428-RA, gene ID in Genbank: LOC100121302). Sequence variation at the *period* locus was examined in the southern and northern lines. Twelve primer pairs were designed in order to amplify gene regions covering parts of exon 3, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17 and 18, representing a subset of the 20 exons present in *period* (appendix 4). These regions were sequenced for male individuals from the northern and southern lines (appendix 3) and sequences were aligned using the software for molecular evolutionary genetic analysis MEGA 5 (Tamura *et al.*, 2011). Synonymous and non-synonymous SNPs were distinguished based on the PERIOD protein sequence as reference (NCBI ID: XP_001604906.2) and haplotypes were constructed using non-synonymous SNPs. In a similar way, the *cycle* locus (NasoniaBase reference NV13263-RA, gene ID in Genbank: LOC100118796) was investigated with 7 primer pairs covering regions of exon 2, 4, 8, 9, 10, 11, 12, 13 and 14 (appendix 4).

Crosses

In order to test the association of the *period* haplotypes with diapause induction, crosses between and within lines were performed to generate F1 females. Virgin females from the southern (denoted “SS”) and northern line (denoted “NN”) were collected as pupae from host puparia in the mass culture and isolated in cotton-plugged 60 mm x 10 mm plastic tubes together with males from the same line or the other line (denoted “S” and “N”). After eclosion and mating, new hosts were provided to the females

for oviposition. The hosts containing developing offspring were kept at 20°C and continuous light until emergence after 3 weeks. After genotyping the parental individuals (appendix 3), the *period* genotype of the F1 female offspring was determined and individuals of each genotype were tested for their photoperiodic diapause induction following the diapause phenotyping protocol described in chapter 3 (repeated hosting for 10 days). Between 35 and 42 females from the intra-line crosses and 69-100 females from the inter-line crosses were screened. The statistical analysis was performed using the R statistical software (R Development Core Team 2012). Survival analysis was used to analyse the switch point for diapause induction. All phenotypic data were analysed using Cox proportional hazard models followed by *post hoc* multiple comparison analysis (packages *survival* and *multcomp* in the R language).

***Natural polymorphism in* period**

The natural variation of *period* haplotype frequency was investigated by screening 119 individuals from seven European populations for which photoperiodic diapause induction was described to follow a latitudinal cline (chapter 2, this thesis). DNA was isolated from individual females (previously stored in 70% ethanol and -20°C) using the standard high salt-chloroform protocol (Maniatis *et al.*, 1982). Two *period* primer pairs were used to amplify two representative regions of the gene covering 88 nucleotides of exon 3 and 102 nucleotides of exon 16 (primers Nv_*per*5 and Nv_*per*11 in appendix 4). The obtained sequences were analysed using the software for analysis of DNA polymorphism data DNAsp5 (Librado & Rozas, 2009) and individual female haplotypes were inferred

from the diploid sequences using the algorithm PHASE (Clark, 1990) implemented in DNAsp. For all individuals, two haplotypes could be distinguished (diploid females) allowing haplotype frequency to be estimated for each population. Linear regression (package *lm* in the R language) was used to test the latitudinal cline in allele frequency in exon 3 and exon 16 and in haplotype frequency. Given that phenotypic data on switch point for diapause induction was available for the sequenced individuals, the correlation between *period* haplotype frequency and mean switch point in each population could be tested with a correlation test. Finally, survival analysis was used to test the difference in switch point between different genotypes at the *period* locus in sequenced individuals from natural populations.

RESULTS

Period *haplotypes*

Using 13 primer pairs for *period*, around 4 Kb were amplified and sequenced for male individuals from the northern and the southern isofemale line. Twenty-one non-synonymous SNPs were identified in 9 exons, yielding three *period* haplotypes (Table 4.1). One haplotype, per_S , was found in the southern line, and two haplotypes (per_{N1} and per_{N2}) in the northern line (Table 4.1). These three haplotypes could be defined based on two focal SNPs: one at the beginning of exon 3 and one in exon 16. The SNP (G/T) in exon 3 leads to the amino acid polymorphism Glycine/Valine and the SNP in exon 16 (A/G) involves an Histidine/Arginine

polymorphism (Table 4.1, Fig. 4.1). The southern haplotype per_S and the northern haplotype per_{N1} are identical for all tested SNPs except for the two SNPs in exon 3 and 16. In contrast, the northern haplotype per_{N2} is unique for all tested SNPs except for the SNP in exon 3, for which it shares the G with the southern haplotype per_S and the SNP in exon 16, for which it shares the G with the northern haplotype per_{N1} (Table 4.1). These two SNPs and their combination were selected as markers for subsequent analysis of *period* haplotypes.

In the sequence of the *cycle* gene only one synonymous SNP was detected in exon 2, differentiating northern and southern lines. Therefore, further analysis focused on the *period* gene.

Table 4.1: Haplotypes of *period* in southern and northern lines based on non-synonymous SNPs in 8 exons. Dots mean that the nucleotide is the same as the one in the first line at the same position. The highlighted SNPs in exon 3 and exon 16 are used to define the three haplotypes.

	Exons																						
Haplo type	3	10			11		12				13			14	15		16		17				
per_S	G (Gly)	G	G	C	T	T	A	C	C	T	G	T	A	T	G	C	A (His)	A	T	C	G		
per_{N1}	T (Val)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G (Arg)	.	.	.	.		
per_{N2}	. (Gly)	A	T	T	C	G	C	A	A	C	C	A	C	C	T	G	G (Arg)	G	C	T	A		

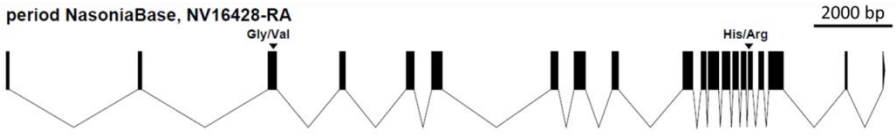

Figure 4.1 Schematic presentation of *period* in *Nasonia vitripennis*. Exons are indicated with boxes and introns with lines. The total length of the gene is 22.4Kb. The positions of the focal SNPs in exon 3 and exon 16 are shown.

Crosses

In order to investigate the association between the observed *period* haplotypes and variation of photoperiodic diapause response, laboratory crosses between northern and southern lines were performed to generate females with different genotypes for which the switch point was measured. Homozygous per_{N1N1} females showed a significantly earlier switch point (6.77±0.2 days) than homozygous per_{N2N2} females (7.62± 0.21 days) (*post hoc* multiple comparison for survival analysis, $P < 0.05$) (Fig. 4.2). Heterozygous per_{N1N2} females showed a switch point (6.51±0.27 days) significantly different from that of homozygous per_{N2N2} (*post hoc* multiple comparison for survival analysis, $P < 0.05$) but not from per_{N1N1} females (*post hoc* multiple comparison for survival analysis, $P = 0.99$) indicating dominance of per_{N1} over per_{N2}. This result suggests that sequence variation in *period* is associated with different diapause responses (haplotype per_{N1} and per_{N2} associated with early and late switch points, respectively). This was confirmed in the interline crosses where heterozygous per_{N1S} showed an earlier switch (7.13±0.15 days) compared to the per_{N2S} females (8.23±0.20 days) (survival analysis, cox model $\chi^2 = 10.22$, $P < 0.01$) (Fig 4.2). The switch points of the per_{N1S} and per_{N2S} females

were both earlier than those of per_{SS} females (12.54±0.54 days) described in chapter 3 and were similar to the homozygous per_{N1N1} and per_{N2N2}, respectively (post-hoc multiple comparison for survival analysis, per_{N1N1} and per_{N1S} P = 0.13; per_{N2N2} and per_{N2S} P = 0.17). A partial dominance of both N haplotypes over S haplotype is thus observed. Overall, these results confirm and refine the findings of the QTL analysis in chapter 3, by showing an association between amino acid polymorphism in the *period* gene and variation in photoperiodic diapause induction.

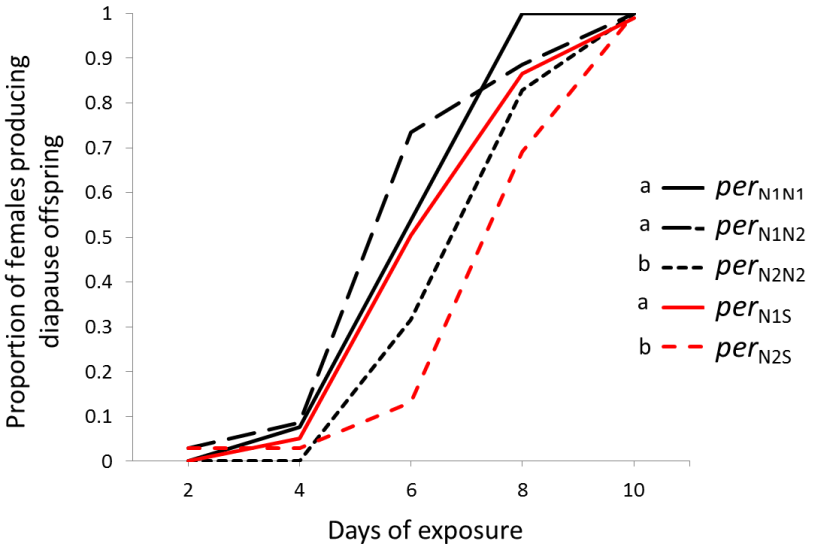

Figure 4.2 Diapause response of *Nasonia vitripennis* females with different *period* genotypes under light:dark cycle 14:10. Black lines represent the response of F1 females from intra-line crosses (northern) which generated three different *period* genotypes: per_{N1N1}, per_{N1N2}, per_{N2N2}. Red lines represent the responses of females from inter-line crosses (northern x southern) which generated two heterozygous genotypes: per_{N1S} and per_{N2S}. Letters in the legend indicate significant difference (*post hoc* multiple comparison for survival analysis, $P < 0.05$).

Natural variation in* period *polymorphism

The abovementioned association between amino acid polymorphism in *period* and photoperiodic diapause induction, prompted us to investigate the natural allelic variation of *period* in individuals from *N. vitripennis* populations collected along a North-South geographic gradient in Europe. Phenotypic data on photoperiodic diapause are available for these populations and show a significant latitudinal cline in switch point (chapter 2, this thesis). The regions containing the non-synonymous SNPs in exon 3 and exon 16, previously identified in two isofemale lines, were amplified and sequenced in 119 individual females from 7 locations. A first analysis of the frequency of the two SNPs in the full dataset, including sequences from all individuals, yielded an overall frequency of the Gly variant in exon 3 of 0.61 and of the Val variant of 0.39. Similar frequencies were found for the two variants in exon 16: the His variant occurred at a frequency of 0.60 and the Arg variant at 0.40. The frequencies of the two SNPs were subsequently compared between populations (Fig. 4.3) and there was a significant latitudinal cline in frequency for alleles in exon 3 (linear regression: $F_{1,5} = 62.85$, $P = 0.0005$, adjusted $R^2 = 0.91$) and exon 16 (linear regression: $F_{1,5} = 29.99$, $P = 0.002$, adjusted $R^2 = 0.83$). The linkage disequilibrium between the two polymorphic sites was high (standardized linkage disequilibrium D' = 0.905 (Lewontin, 1964), Fisher's exact test $P < 0.001$) and the *period* haplotypes were defined based on the combination of the variants at the two focal SNPs alone.

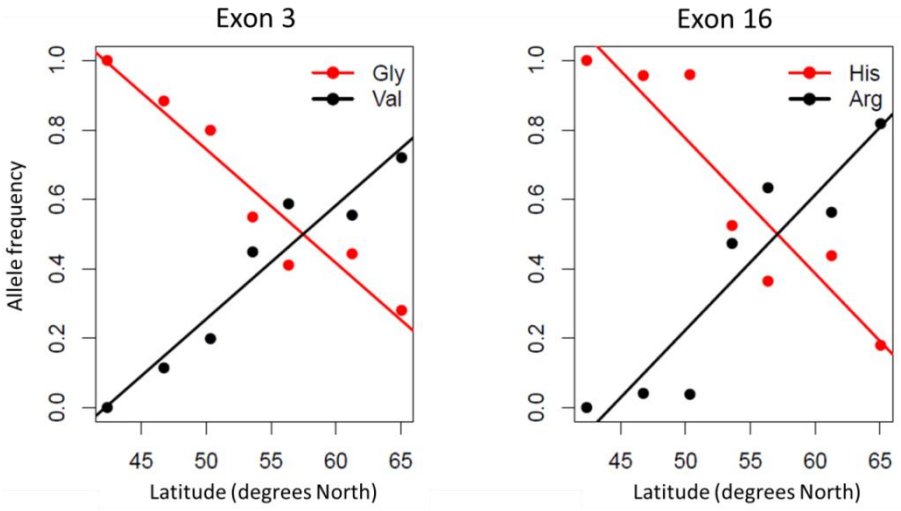

Figure 4.3 Latitudinal cline in SNP frequency in two non-synonymous SNPs in exon 3 and exon 16 in the *period* gene of *Nasonia vitripennis*.

The haplotypes per_S and per_{N1} accounted for most of the variation, as their frequencies in the entire dataset were 0.58 and 0.34, respectively. Haplotype per_S was present in all populations with a decreasing frequency towards northern latitudes (linear regression: $F_{1,5}$ = 29.18, P = 0.002, adjusted R^2 = 0.82). In contrast, haplotype per_{N1} showed an opposite cline with increasing frequency in northern populations (linear regression: $F_{1,5}$ = 21.28, P = 0.006, adjusted R^2 = 0.77) and was absent in the two most southern populations COR and SWI (Fig. 4.4). The haplotype per_{N2} was rare and observed at low frequencies in northern populations only (total frequency 0.05). A fourth haplotype per_x (exon 3: Val, exon 16: His) was found, present at low overall frequency of 0.02 in only two populations.

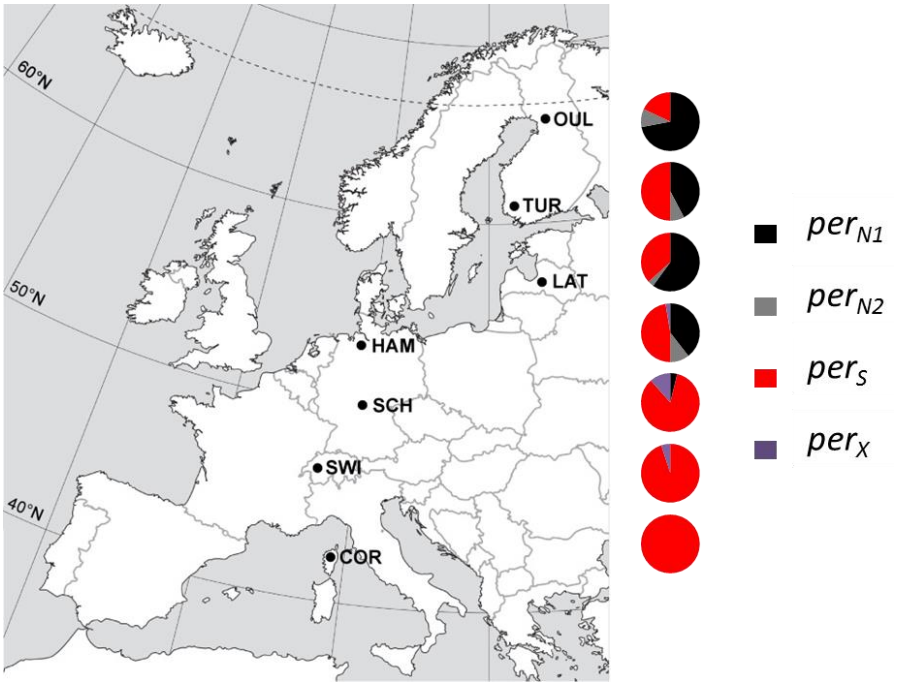

Figure 4.4: Occurrence of four *period* haplotypes in *Nasonia vitripennis* populations from seven locations in Europe. Sectors of the pie charts represent the proportion of each haplotype.

Period *polymorphism and diapause induction*

The observed haplotype frequencies of per_S and per_{N1} in populations across the North-South gradient correlate with maternal photoperiodic diapause induction, measured as population mean switch point at LD 14:10 (chapter 2) (for per_{N1}, Kendall's rank correlation tau = -0.68, $P < 0.05$; for per_S, Kendall's rank correlation tau = 0.61, $P = 0.07$) (Fig. 4.5).

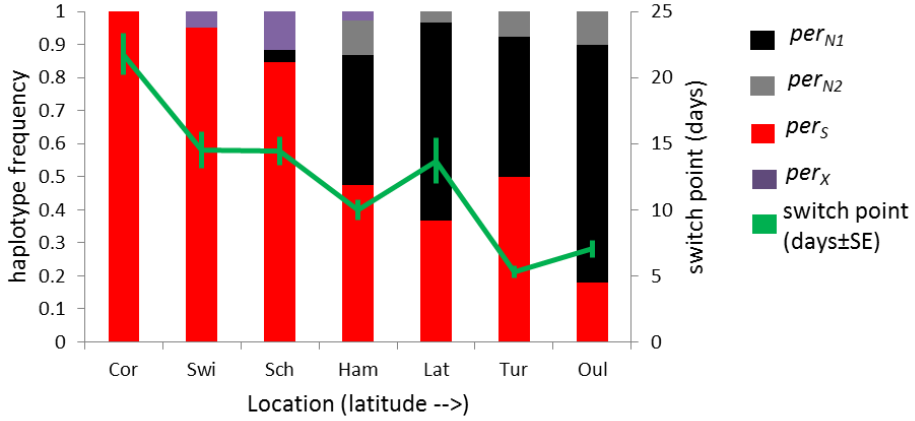

Figure 4.5: Latitudinal cline of *period* haplotype frequency in *Nasonia vitripennis* natural populations from seven locations in Europe and correlation with mean population switch point for diapause induction measured under light:dark 14:10 (chapter 2). Only females that reached the switch were considered for the calculation of the mean population switch point.

Since haplotypes per_{N1} and per_{S} are the most common ones, 88% (105 out of 119) of the investigated females were homozygous or heterozygous for these two haplotypes (proportions in full dataset: 0.25 for $per_{N1N1,}$ 0.13 for per_{N1S}, 0.5 for per_{SS}). The mean switch points for this subset of individuals were 9.20±0.67, 7.14±0.83 and 16.04±1.09 days for $per_{N1N1,}$ per_{N1S} and per_{SS}, respectively (survival analysis, cox model effect of genotype on switch point χ^2 = 39.06, $P < 0.01$). These values resemble the values obtained for homozygous and heterozygous individuals in our controlled crosses between northern and southern lines and confirm the dominance of haplotype per_{N1} over per_{S} and the different effect of *period* polymorphism on diapause (*post hoc* comparison for survival analysis: per_{N1N1} and per_{N1S} $P = 0.19$; per_{N1N1} and per_{SS} $P < 0.01$; per_{N1S} and per_{SS} $P < 0.01$) (Fig. 4.6).

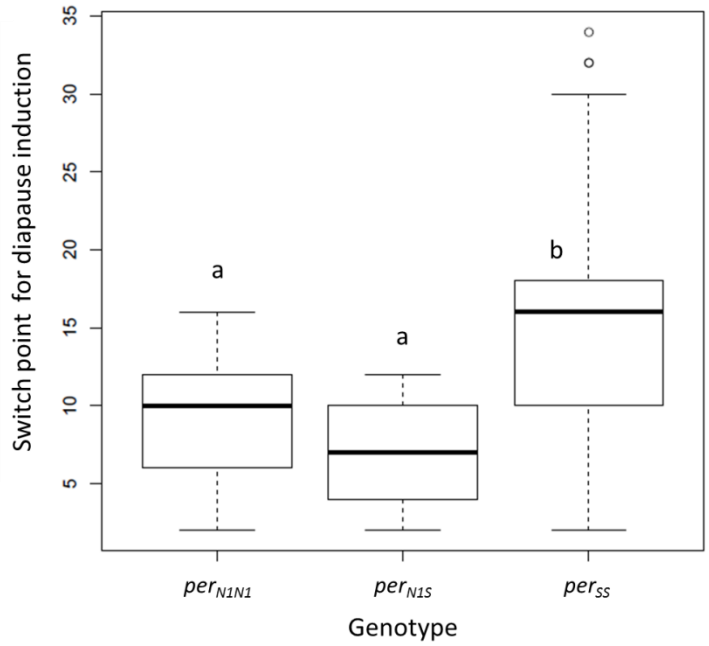

Figure 4.6: Diapause switch point in *Nasonia vitripennis* females with different *period* genotypes. Letters indicate significant differences (*post hoc* multiple comparison for survival analysis). Individuals from seven European locations are grouped based on their genotype at the *period* locus. Switch point for each female was measured under light:dark 14:10 (chapter 2). Females that did not reach the switch point were excluded.

It was not possible to statistically correlate *per* genotypes and diapause switch point within each population, given the low sample size for some genotypes. Nevertheless, some trends can be observed (Table 4.2): in southern populations (COR, SWI and SCH) there is little variation in *per* haplotypes, so it is not possible to compare the response of individuals from different genotypes. The intermediate populations, HAM and LAT, show late switch points for per_{SS} individuals and earlier ones for individuals with northern haplotypes in line with previous results (Table 4.2). This

correlation does not seem to be present in northern populations (TUR and OUL) were the switch point is early also in the homozygous per_{SS} individuals (Table 4.2). These results suggest that *period* is not the only gene involved in photoperiodic diapause response, but that the genomic background is also important, possibly with the involvement of several other genes.

Table 4.2: Mean switch point (days ± SE) for diapause induction in *Nasonia vitripennis* populations from seven locations in Europe, separated based on their genotype at the *period* locus. Only individuals that reached the switch point are considered.

		Mean switch point (days±SE)		
	Latitude (°N)	per_{N1N1}	per_{N1S}	per_{SS}
OUL	65.06	5.0±0.96	6.0±na*	5.3±0.6
TUR	61.26	8.0±2.0	5.4±0.8	8.0±1.1
LAT	56.36	10.7±1.5	8.0±4.0	22.0±4
HAM	53.59	10.4±1.3	10.0±1.1	11.2±1.2
SCH	50.32	na	10.0±na*	13.3±1.8
SWI	46.75	na	na	14.0±1.6
COR	42.37	na	na	21.8±1.6

*sample size =1

DISCUSSION

In this chapter we demonstrate that genetic variation in the clock gene *period* is associated with adaptive variation in photoperiodic diapause induction in *Nasonia vitripennis*. We found a latitudinal cline in *period* allele and haplotype frequency that correlates with a natural gradient in switch point for diapause induction. These results indicate a link between the circadian and seasonal clock and suggest a role for the clock gene

period in local adaptation to seasonal cycles through its involvement in photoperiodic diapause.

In the first experiment, fragments of the *period* gene in northern (Oulu, Finland) and southern (Corsica, France) lines were sequenced and three different haplotypes were identified based on non-synonymous SNPs in coding regions leading to amino acid substitutions. Two main SNPs in exon 3 and exon 16 represent the most relevant modifications associated with variation in phenotype, however it is not known whether these amino acid substitutions lead to a structural change in the PER protein that influences its function. The full sequence of the entire *period* gene could reveal more functional SNPs in exons or regulatory regions that might affect the function or expression level of *period* that in turn influence the diapause response. Obviously, knowledge on the detailed physiological mechanism of photoperiodic induction of diapause would reveal the exact role of *period* and its encoded protein, which will facilitate the understanding of the functional link between the observed genetic variation and phenotypic adaptive variation. Given the correlation between *period* polymorphism and switch point for diapause induction, one hypothesis is that *period* is involved in the threshold mechanism that governs photoperiodic diapause in *Nasonia* (chapter 2, this thesis). Different *period* variants could affect the threshold level of required light:dark cycles or the rate of accumulation of the information from the photoperiodic cue resulting in both case in variable diapause response expressed as different switch point. These hypotheses are further discussed in chapter 6.

Our screen of natural populations for sequence variation in *period*

revealed a latitudinal cline in *per* haplotype frequencies. This cline correlates with the latitudinal cline in photoperiodic diapause induction previously documented for these populations of *N. vitripennis*. The haplotype that was most frequent in the south, termed haplotype per_S, decreased towards northern latitudes, but was still present in northern populations at low frequencies. The northern haplotype per_{N1} showed the opposite latitudinal cline with decreasing frequencies towards low latitudes, being completely absent in the two most southern populations. The haplotypes per_{N2} and per_X were rare and their frequency did not show any latitudinal cline. Natural selection along latitudinal geographic gradient is expected to result in the proper timing for diapause response at each latitude through the selection of the "best" photoperiodic counter (threshold level of photoperiodic cycles inducing diapause response). Genes involved in the counter machinery are thus under selection and are expected to show correlated polymorphisms, as in the case for *period*. Clinal variation in allelic frequency can also be the result of neutral processes (e.g. genetic drift), however our previous analysis of the genetic differentiation between these populations along the North-South gradient, measured with neutral microsatellite markers, showed a degree of differentiation that did not correlate with latitude (Paolucci *et al*., 2013; chapter 2, this thesis). This suggests that the latitudinal cline in *period* polymorphism results from selection pressure at this locus due to environmental conditions along a North-South gradient. This gradual selection does not affect neutral loci used for the population differentiation analysis (discussed in chapter 2).

As described in the introduction, latitudinal clines in clock gene

polymorphisms have been documented for *Drosophila melanogaster* in which different variants of *period* and *timeless* are geographically distributed according to latitude. Unfortunately, there are no published data on latitudinal variation in diapause in European *D. melanogaster* populations. As the observed polymorphism cannot be correlated with diapause response of natural populations, it is not possible to interpret its functional significance in terms of photoperiodic adaptation. However, Sawyer *et al.* (1997) have proposed that the northern allele $(Thr\text{-}Gly)_{20}$ might be adaptive at higher latitude as it has an effect on the stabilization of circadian rhythmicity. Flies carrying the $(Thr\text{-}Gly)_{20}$ allele showed no difference in circadian period measured under two temperatures. This property allows the flies to maintain a functional internal clock in northern environments characterized by large temperature changes. Fabian *et al.* (2012) screened latitudinal variation in allelic frequency in many candidate genes in three populations of *D. melanogaster* in North America which exhibit clinal variation in a number of important life history traits, including diapause. Among others, they found clinal differentiation in the clock genes *timeless*, *timeout*, *cryptochrome* and *clock*. Interestingly, they did not find a clinal polymorphism in *period* possibly due to the stringent criteria for defining candidate SNPs (Fabian *et al.*, 2012). Recently, genetic variation in the clock gene *cryptochrome* was also investigated in natural European populations of *D. melanogaster*. Although an adaptive explanation for the observed polymorphism was suggested, there was no latitudinal cline in allelic frequency (Pegoraro *et al.*, 2014). In vertebrates there have also been a few studies on clock gene polymorphism. For example, in Pacific salmonids of the genus *Oncorhynchus*, the length

variants of the functionally important polyglutamine repeat motif (PolyQ) in the gene *Clock* was evaluated under different environmental conditions along a latitudinal gradient. The *Clock* polymorphism showed contrasting latitudinal clines in four salmonid species with overlapping breeding range: in chum salmon the 335 bp allele increased in frequency with increasing latitude, differently from chinook salmon where the same allele decreased in frequency towards higher latitudes (O'Malley *et al.*, 2010). This suggests that the functional association between breeding latitude and the *Clock* polymorphism is not obvious and the seasonal breeding regulated by photoperiod might be based on different mechanisms in different species (O'Malley *et al.*, 2010). More intraspecific and interspecific comparative studies of clock gene polymorphism along latitudinal gradients (e.g. similar clinal variation in distant geographic locations) are necessary. They should be accompanied with observations on variation in photoperiodic response, as this will help to shed light onto the genetic basis of adaptation to different seasonal environments and on the role of the circadian clock.

Looking on a smaller scale, the correlation between *per* genotype and diapause tendency was not obvious. In northern populations (TUR and OUL) the individuals with southern haplotypes showed an earlier switch point compared to individuals with the northern haplotypes, opposite to the expected direction. This observation suggests that *period* is not the only gene involved in photoperiodic diapause (in line with a multi-genic basis revealed by QTL analysis in chapter 3) and its role in regulating diapause response is affected by interaction with other genes and genomic background. An introgression experiment in which the different *per* alleles are crossed into a common genetic background may help to

further determine the role of *period* in diapause and the role of other relevant genes. Also, it will be important to characterize the molecular clock mechanism of *Nasonia* in order to clarify in which component of the clock *period* is involved and how that interacts with diapause response.

This is the first study of clinal variation in the *period* gene that correlates it with the quantitative photoperiodic response in terms of diapause. Our findings indicate that *period* is involved in the molecular mechanism of the photoperiodic counter, which is the focal target of natural selection (chapter 2, this thesis). However, the exact mechanism by which *period* affects diapause remains elusive. There are several possibilities: *period* might act on photoperiodism as isolated gene and could, for example, behave as a pleiotropic gene affecting different traits such as circadian rhythmicity and photoperiodic diapause. Alternatively, *period* can affect diapause through its role in the circadian clock unit which would affect photoperiodic response as a module (Emerson *et al.*, 2009). To investigate these possibilities, it will be crucial in the future to characterize the molecular circadian clock mechanism of *Nasonia* and the function of *period* and other genes in the clock.

Chapter 5

A test for variation in free running period and circadian locomotor activity in *Nasonia vitripennis*: is there a latitudinal cline?[3]

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Lucia Salis
Monique Suelmann
Louis van de Zande
Leo W. Beukeboom

[3] Part of the experiments presented in this chapter were performed by Lucia Salis at the Institute of Entomology, České Budějovice, CZ. I would like to thank Dr. David Doležel (head of the Laboratory of Molecular Chronobiology) for hosting Lucia Salis. I would also like to thank Dr. Roelof Hut (Chronobiology group, Centre for Behaviour and Neurosciences, University of Groningen, NL) for helpful discussions on the experimental design and analysis of the results

ABSTRACT

Endogenous circadian rhythms are ubiquitous and regulate many physiological processes of living organisms. Because of its capacity to anticipate daily light:dark cycles, the circadian system is believed to be functionally involved in photoperiodic response and to have a shared genetic basis. In the parasitic wasp *Nasonia vitripennis*, natural variation in photoperiodic diapause response is associated with allelic variation of the clock gene *period* which in turn could lead to differences in circadian clock properties. This chapter describes the natural variation in circadian locomotor activity in European populations. An effect of sex and mating status was observed in the proportion of rhythmic individuals: males and unmated individuals are in general more rhythmic than females and mated individuals. In rhythmic virgin females, the free running period *tau* showed a significant shallow latitudinal cline, with longer *tau* towards northern latitudes. A tested northern line (Oulu, Finland) showed a higher level of circadian locomotor activity compared to a southern line (Corsica, France), under constant light conditions and light:dark 12:12. The observed natural variation in properties of the circadian rhythmicity might be the result of natural selection on clock gene allelic variation.

INTRODUCTION

Many organisms, ranging from bacteria to mammals, possess endogenous rhythmicity which is maintained under constant environmental conditions and entrained by external stimuli such as light and temperature to allow synchronization with external daily cycles of environment. Typically, the length of the endogenous rhythm called *free running period* (*tau*, τ), is approximately 24 hours. The intrinsic biological rhythms evolved as adaptation to the environmental cycles generated by the rotation of the Earth around the sun, which affects many abiotic factors such as daily light. The endogenous circadian clock allows organisms to anticipate the external daily cycles of the environment, facilitating the synchronization with environmental cycles. In addition, it has the intrinsic value to coordinate internal cyclic physiological processes which occur also in the absence of external stimuli (Vaze & Sharma, 2013).

A key role for the internal circadian clock is related to photoperiodism (Yerushalmi & Green, 2009; Koštál, 2011). The circadian clock is able to measure the length of the photoperiod and is therefore believed to be involved in the seasonal regulation of life cycle characteristics, although this hypothesis is not equivocally accepted (Bradshaw & Holzapfel, 2010). As shown in many species, seasonal photoperiodic responses differ geographically as result of variation in local photoperiodic conditions signalling seasonal changes. The necessity to respond differently to photoperiodic cues in various environments might require different properties of the circadian clocks used to measure the daily light:dark cycles (Hut & Beersma, 2011). Investigating geographic variation in circadian systems contributes to the understanding of the role

of internal circadian clocks in photoperiodism. In the model plant species *Arabidopsis thaliana* an increase in *tau* towards northern latitudes has been observed, coinciding with clinal variation in seasonal flowering time regulated by photoperiodic cycles (Michael *et al.*, 2003). This was explained as an adaptive response and has been corroborated by experimental manipulation of circadian properties in *Arabidopsis* (Yerushalmi *et al*., 2011). In insects, latitudinal clines in properties of circadian clocks have been reported for *Drosophila* species (reviewed in Hut *et al.*, 2013). In *Drosophila auraria* (Pittendrigh & Takamura, 1989) and *D. ananassae* (Joshi, 1999), the period of the endogenous clock was measured for locomotor activity and eclosion, respectively, and was found to be positively correlated with latitude. However, in *D. littoralis* and *D. subobscura*, opposite latitudinal clines for timing of eclosion were found (Lankinen, 1986, 1993), with shorter *tau* in northern populations. Other important examples of clinal variation of circadian clocks are related to genetic polymorphisms found in the clock genes *period* and *timeless* in *Drosophila melanogaster* (Kyriacou *et al*., 2008). These few examples suggest that variation in endogenous circadian clocks may confer adaptive advantages to populations dealing with different environmental conditions.

In the previous chapters of this thesis it was shown that natural variation in photoperiodic diapause response in the parasitoid *Nasonia vitripennis* is associated with the clock gene *period* and that genetic polymorphism in this gene follows a similar latitudinal cline as the variation in switch point for diapause response. This indicates that the circadian clock in *N. vitripennis* is involved in the photoperiodic seasonal

response. In this chapter we investigate variation in circadian activity in *N. vitripennis*, by measuring the free running period of locomotor activity in isofemale lines originating from populations collected along a North-South gradient. We further investigate variation in the level of activity under different conditions in two isofemale lines from southern (Corsica, France) and northern (Oulu, Finland) latitudes that were previously used for genetic analysis of photoperiodic diapause response.

MATERIALS AND METHODS

Experimental lines

To study the variation in locomotor activity in natural populations of *Nasonia vitripennis*, we used the isofemale lines established from field material, previously involved in the latitudinal clinal studies presented in chapter 2 and chapter 4. The lines originated from seven European sampling locations (OUL (Finland, Oulu): 65°3’40.16”N, 25°31’40.80”E; TUR (Finland, Turku): 61°15’40.53”N, 22°13’23.96”E; LAT (Latvia): 56°51’22.56”N, 25°12’1.38”E; HAM (Germany, Hamburg): 53°36’23.62”N, 10°10’17.74”E; SCH (Germany, Schlüchtern): 50°19’56.10”N, 9°30’47.00”E; SWI (Switzerland): 46°44’9.14”N, 7°6’57.34”E; COR (France, Corsica): 42°22’40.80”N, 8°44’ 52.80”E). The lines were maintained on *Calliphora* spp pupae as hosts in mass culture vials under diapause-preventing conditions (long photoperiod, 20–25 °C). For this study we used between 17 - 25 isofemale lines for each location and 4-8 individuals for each isofemale line for a total number of 1512 individuals (797 females and 715

males). However, while recording the locomotor activity during the course of the experiment, some individuals died before sufficient data could be collected for subsequent analysis of free running period and rhythmicity. Thus, the total number of analysed individuals was in the end 1072: 548 females (163 virgin and 385 mated) and 544 males (122 virgin and 402 mated).

The level of activity under different conditions (see below) was investigated in mated female individuals from two selected isofemale lines originating from Oulu, Finland ("northern" line) and Corsica, France ("southern" line). These lines were previously used for the genetic analysis of photoperiodic diapause response presented in chapters 3 and 4.

Locomotor activity measurement and free running period

The locomotor activity measurements were performed using the Trikinetics monitoring system which is specifically designed to record locomotor activity in *Drosophila* flies. One of the purposes of this study was to adapt the *Drosophila* monitoring system to *Nasonia vitripennis* wasps. The wasps for the experiment were collected one day after emergence (mated group) or collected as pupae (virgin group) and transferred individually into glass tubes. One side of each tube was closed with melted wax and the other side was closed with a cotton plug containing a sugar-water solution. The tubes containing the mated individuals were immediately placed into the Trikinetics activity monitors (32 tubes per monitor) and transferred into incubators under controlled conditions. The wasps collected as pupae were allowed to develop into adults at room temperature and subsequently placed into the monitors to

start the experiments (virgin group). The experimental conditions included 4 days of entrainment period under 16:8 hours light:dark cycle followed by constant darkness for 10 days (with constant temperature 20°C). The monitors were connected to a computer and the raw locomotor activity data were collected and stored through the software DAMSystem (supplied with the Trikinetics system). At the end of the experiment, data were retrieved and analysed using the software MATLAB (MATLAB, The MathWorks Inc., Natick, 2000). For the analysis of rhythmicity and the length of the free running period, the first four days of entrainment under light:dark conditions were excluded. Data from the fifth day onwards were used to determine whether a wasp was rhythmic or not, based on the possibility to detect significant periodicity across several consecutive days. For rhythmic individuals, the intrinsic free running period *tau* was calculated through periodogram analysis based on a time interval of 30 minutes.

The effect of location, latitude, sex, and mating status on the percentage of rhythmic individuals was statistically tested using a generalized linear mixed effect model in which the random factor was isofemale line nested into location (package *lme4* in the statistical software R). Linear mixed effect models were used to test the effect of location, latitude, sex and mating status on the length of the free running period *tau*, with isofemale line nested into location as random effect (package *nlme* in the statistical software R)

Analysis of activity level in northern and southern line

The TriKinetics *Drosophila* activity monitor system was also used to measure the level of locomotor activity in individuals from the selected northern and southern lines. In the first experiment, 23 females from the southern line and 20 females from the northern line were isolated in glass tubes closed at each side with a cotton plug and a drop of honey. The tubes were placed in monitors and the monitors transferred in light-controlled wooden boxes, placed inside a temperature-controlled climate chamber. The wasps were exposed to an initial entrainment period of 3 days at light: dark cycle 16:8, followed by constant light conditions (with constant temperature=20°C). As described above, the data could be retrieved from the monitoring system through the DAMSystem software. The activity level of each individual was defined as an average of the locomotor activity based on 60 minutes/bin. In the second experiment, 16 wasps from the northern line and 16 from the southern line were exposed to 12:12 light:dark conditions and activity was measured during light and dark phase.

We attempted to investigate the activity level in *Nasonia vitripennis* females before the switch point for diapause induction (when they produce normal developing offspring) as well as after the switch point (when they produce diapausing offspring). In order to determine the switch point of the tested individuals, we had to expose the individuals to short photoperiods to induce diapause and we had to simultaneously provide host pupae to each individual, so that eggs could be laid and diapause scored in the offspring (chapter 2 for diapause phenotyping). Twenty-five females of the southern line and 20 of the northern line were

used for this experiment. They were placed in tubes that were closed on both sides with a cotton plug and honey, but every other day one host pupa was provided on one side of the tube during two hours for egg laying. The removed hosts were transferred to constant light and constant temperature of 20°C for subsequent diapause scoring. Activity level was measured as described above (per hour/bin). The number of offspring from parasitized hosts was very limited, probably due to the fact that we left each host for only 2 hours every other day. Therefore, we set up a last experiment in which 25 females from the northern line were placed in the monitor tubes closed with a cotton plug and honey on one side and a host on the other side. The host was replaced every 2 days but it was kept in the monitoring system for 2 full days so that a female would have enough time to lay eggs. Diapause was subsequently scored in the offspring (inside the removed hosts) and switch point was determined. Ten out of 25 individuals reached the switch point for diapause induction during the course of the experiment, so the level of activity before and after the switch could be measured and compared. The difference in activity level was statistically tested with generalized linear models (package *stats* in the statistical software R)

RESULTS

Rhythmicity in natural populations of Nasonia vitripennis

We studied the rhythmicity in locomotor activity under constant darkness in *Nasonia vitripennis* individuals, after an entrainment period of four days

under light:dark 16:8. Figure 5.1 shows representative examples of double-plotted actograms (48 hours) which display the activity of single individuals during the monitoring period. Most male individuals (90%) used in the experiment showed rhythmic locomotor activity), differently from females of which only 68% were rhythmic (generalized linear mixed effect model, effect of sex: χ^2 = 65.71, P < 0.01). The mating status also significantly affected the rhythmicity: 94% of virgin individuals were rhythmic but only 73% of mated individuals (generalized linear mixed effect model, effect of mating status: χ^2 = 112.39, P < 0.01). In females, only 58% of mated individuals were rhythmic compared to 92% of virgins (generalized linear mixed effect model, effect of mating status within females: χ^2 = 50.25, P < 0.01). In males, the effect of mating status was smaller: 97% of virgin males were rhythmic and the proportion of rhythmicity among mated individuals was 88% (generalized linear mixed effect model, effect of mating status within males: χ^2 = 12.18, P < 0.01) (Fig. 5.2). However, the statistical analysis did not reveal a significant interaction effect between mating status and sex (generalized linear mixed effect model, effect of mating status x sex: χ^2 = 1.57, P = 0.20).

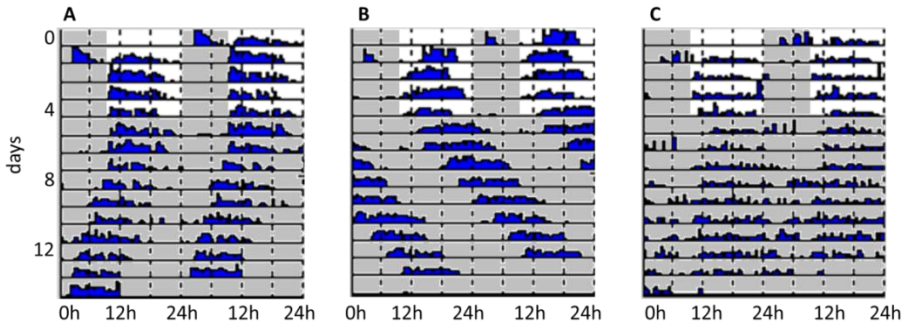

Figure 5.1 Representative double-plotted actograms for *Nasonia vitripennis* wasps. Grey background corresponds to dark hours, white background to light hours. Wasps were entrained for the first 4 days under LD 16:8 and then exposed to constant darkness. **A)** Rhythmic male with τ < 24h. **B)** Rhythmic female with τ > 24h. **C)** Arrhythmic female.

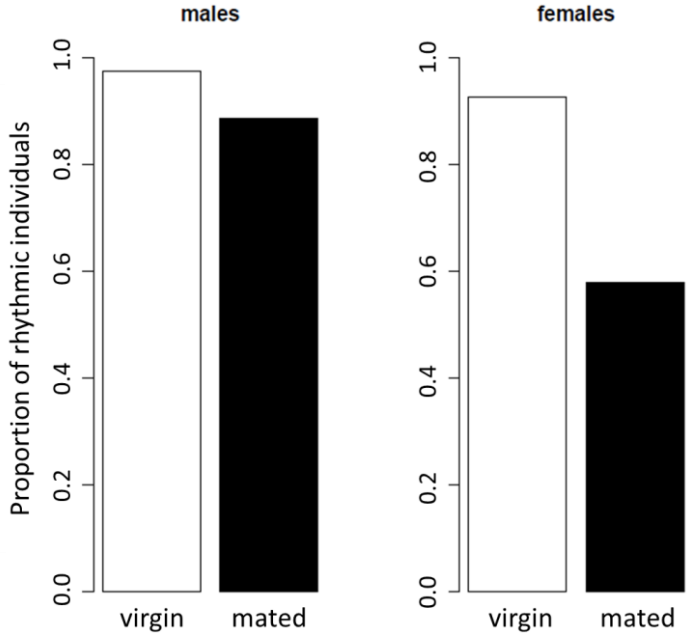

Figure 5.2 Overall proportion of rhythmicity in *Nasonia vitripennis* individuals measured as internal rhythmicity under constant darkness

The overall proportion of rhythmic individuals in each population ranged from 75% in the SWI population to 84% in the COR population and the interaction between location and sex was significant (generalized linear mixed effect model, effect of location x sex: χ^2 = 33.00, P < 0.01). Within each sex, there was a significant effect of location (generalized linear mixed effect model, effect of location: for females χ^2 = 13.28, P < 0.05; for males χ^2 = 13.46, P < 0.05) (Fig. 5.3). Despite the significant effect of location on proportion of rhythmic individuals in the two sexes, we did not find a significant correlation between latitudinal origin of the analysed isofemale lines and proportion of rhythmic individuals (generalized linear mixed effect model, effect of latitude: χ^2 = 1.77, P = 0.18). Similar results with no latitudinal effect were obtained when data were separated by sex (generalized linear mixed effect model, effect of latitude: for females χ^2 = 1.65, P = 0.19; for males χ^2 = 0.16, P = 0.68) and mating status (generalized linear mixed effect model, effect of latitude: for virgin individuals χ^2 = 2.22, P = 0.13; for mated individuals χ^2 = 1.60, P = 0.20). These results indicate that the observed variation between populations in proportions of rhythmic individuals does not follow a latitudinal cline.

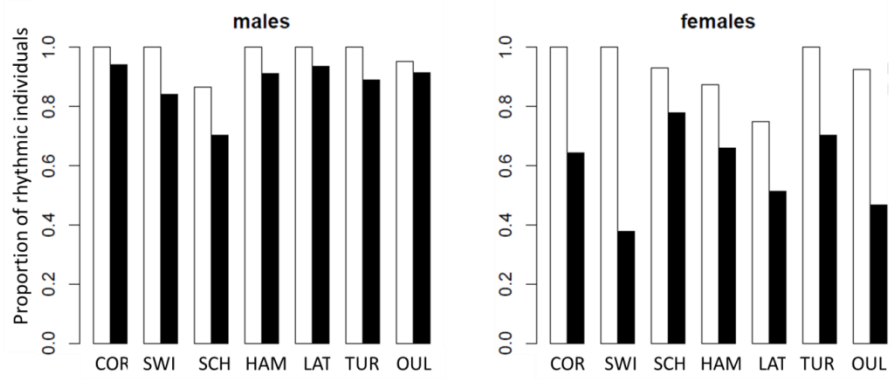

Figure 5.3 Proportion of rhythmic *Nasonia vitripennis* individuals in populations originating from seven locations in Europe. White bars are virgin individuals, black bars are mated individuals.

***Free running period in natural populations of* Nasonia vitripennis**

We analysed the free running period (*tau*) of rhythmic individuals during 10 days under constant darkness. There was a large variation in *tau*, which ranged from 22 hours to 27.5 hours, with an average of 24.16±0.03 hours. Sex, mating status and location had significant effects on *tau* (linear mixed effect model, effect of sex: $LRT = 2.22$, $P = 0.13$; effect of mating status: $LRT = 44.32$, $P < 0.01$; effect of location: $LRT = 38.38$, $P < 0.01$). Overall, females showed a longer *tau* compared to males (24.39±0.04 and 23.97±0.04 hours, respectively) and virgin individuals had longer *tau* compared to mated individuals (24.49±0.05 and 24.00±0.03 hours, respectively). Given the significant effect of the interaction between location, sex and mating status (linear mixed effect model, effect of location x sex x mating status: $LRT = 67.93$, $P < 0.01$), the data were separated in four groups by sex and mating status. The effect of location was significant in virgin females (linear mixed effect model, effect of

location: *LRT* = 14.56, *P* < 0.05) and mated males (linear mixed effect model, effect of location: *LRT* = 13.02, *P* < 0.05) but not significant in mated females (linear mixed effect model, effect of location: *LRT* = 12.02, *P* = 0.06) and virgin males (linear mixed effect model, effect of location: *LRT* = 4.19, *P* = 0.65). Additionally, we observed a significant latitudinal cline in *tau* only in virgin females (linear mixed effect model, effect of latitude: *LRT* = 4.28, *P* < 0.05) (Fig. 5.4). In this group, the free running period was shorter for individuals of southern latitudes and increased towards the north. The slope of the regression was 0.05±0.01 which corresponds to an increase of 3.06 minutes of *tau* for every degree of latitude. In our latitudinal cline covering 23 degrees, such increase would correspond to a difference of 70.38 minutes in *tau* between COR (most southern location) and OUL (most northern location). In fact, the average *tau* we measured for virgin females in COR was 24.6±0.12 hours and for OUL 25.42±0.11 hours corresponding to a difference of 49.2 minutes. Although the cline is rather shallow, the correlation between latitude and *tau* is significant.

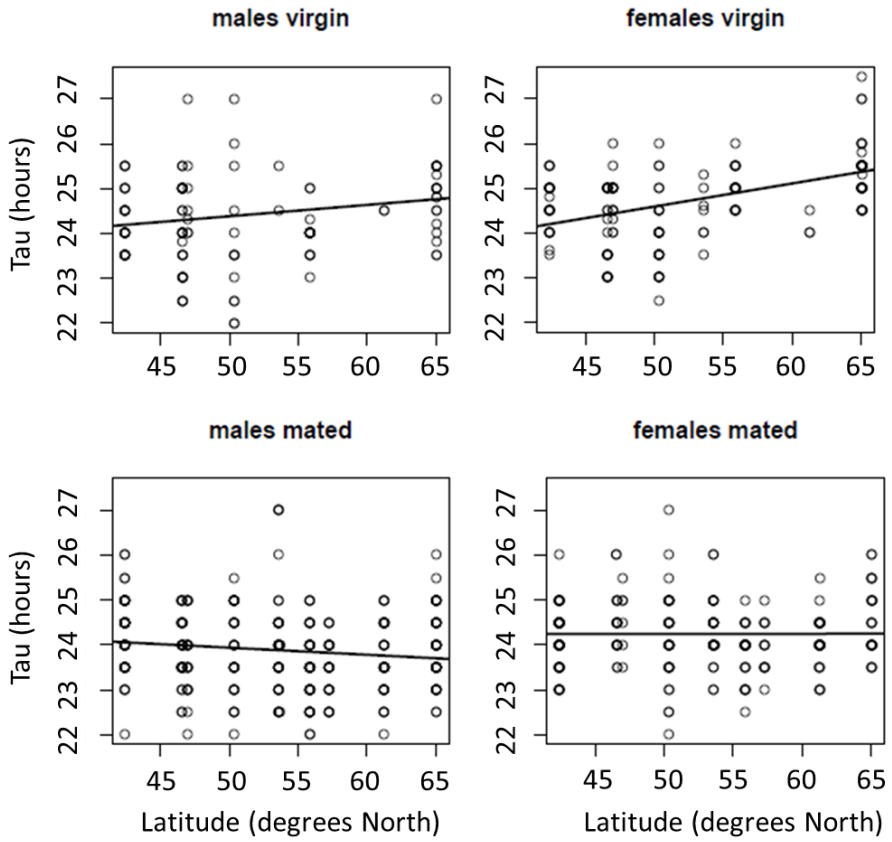

Figure 5.4 Latitudinal cline in free running period for locomotor activity (measured in constant darkness) in natural populations of *Nasonia vitripennis*

Locomotor activity in selected northern and southern lines

In constant light conditions, after an entraining period of three days in light: dark 16:8, females from the northern line were overall highly active, showing an average activity level of 21.54±3.04 per hour bin compared to 5.26±0.8 for the females from the southern line (generalized linear model, effect of line: χ^2 = 43.07, P < 0.01) (Fig. 5.5A). Similar differences in the

activity level were observed in light:dark 12:12. Under this photoperiod, a clear difference in activity is visible between northern and southern lines during the light phase with higher activity level measured for northern females. Individuals from both lines hardly move during the dark phase (generalized linear model, effect of light condition: χ^2 = 23.43, P < 0.01; effect of line: χ^2 = 61.35, P < 0.01) (Fig. 5.5B).

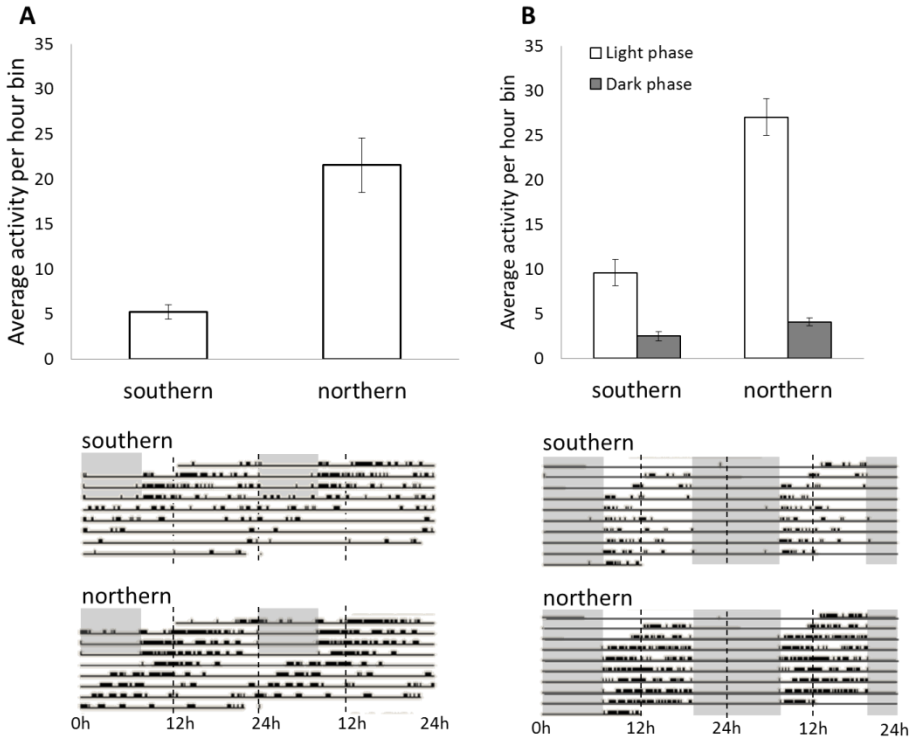

Figure 5.5 Level of activity in the southern and northern selected *Nasonia vitripennis* isofemale lines. Representative double-plotted actograms are shown: grey background corresponds to dark phase, white background corresponds to light phase. **A)** Level of activity measured under constant light after 3 days of entrainment in light:dark 16:8. **B)** Level of activity measured under light:dark 12:12

We attempted to investigate the difference in activity level between females before the switch point for diapause induction and females after the switch point under light: dark 12:12. Firstly, each individual was provided with one host for 2 hours every other day for oviposition, which allowed to measure the effect of the host on the level of activity. We observed an overall increase of activity level in females that received a host for a short period in both the southern and northern line. There was also a significant effect of light condition, with higher level of activity in the light phase compared to the dark phase (generalized linear model, effect of host: $\chi^2 = 122.36$, $P < 0.01$; effect of light condition: $\chi^2 = 102.47$, $P < 0.01$) (Fig. 5.6). During the light phase, northern females that received a host showed an average activity level of 33.58±2.39 per hour bin corresponding to about 19% of increase in activity level compared to females without hosts (27.03±2.05 per hour bin). Similarly, southern females provided with a host had a higher activity (16.28±2.13 per hour bin) than southern females without a host (9.59±1.46 per hour bin). Interestingly, there was a large increase in activity during the dark phase in females with a host for both northern and southern lines. During the dark phase, southern females that received a host moved with a level of 9.14±1.72 per hour bin and northern females with a level of 22.62±2.24, which were both higher than the activity levels during dark phase of females without hosts (2.50±0.57 and 4.10±0.47 per hour bin for southern and northern females, respectively). Overall, the activity of southern females was lower than that of northern females, in line with previous results (generalized linear model, effect of line: $\chi^2 = 80.63$, $P < 0.01$) (Fig. 5.6).

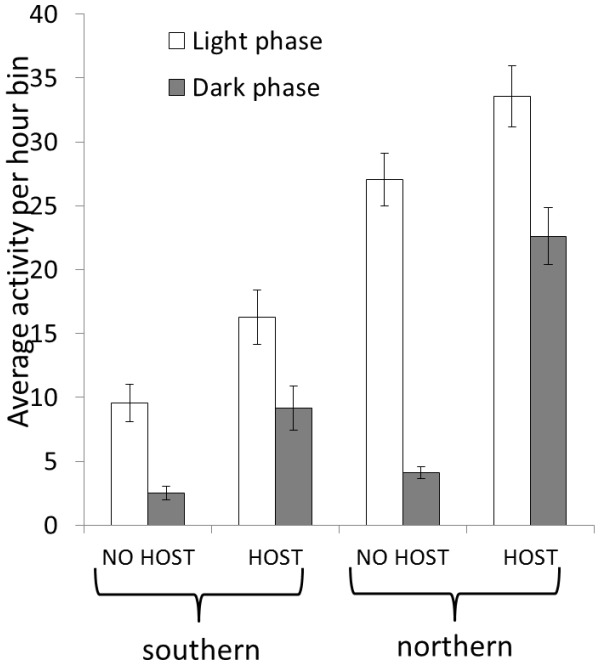

Figure 5.6 Level of activity in the southern and northern selected *Nasonia vitripennis* isofemale lines under light:dark 12:12. Shown are the activity levels of females without host (NO HOST) and females provided with host for 2 hours every other day.

Secondly, we used northern females exposed to the diapause inducing photoperiod 12:12 to measure the level of activity between females producing normal developing larvae and females producing diapausing larvae (before and after the switch point). In the previous experiment not enough offspring were present in the host, probably because the host was available for only 2 hours every other day for oviposition. Therefore in another experiment, females were re-hosted every other day and the host was kept in the tube for 2 full days to allow for a longer oviposition period. Diapause was subsequently scored in the offspring. Overall, we observed an increase in the level of activity in the females that had continuous access to a host compared to the females in the previous experiment that were provided with a host for only 2 hours (activity level during the light

phase for females with continuous access to the host was 53.58±4.63 per hour bin and the activity of females with limited access to the host was 33.58±2.39 per hour bin). Although the level of activity appeared to be lower in females before the switch point (47.48±5.67 per hour bin) compared to females after the switch point (62.74±7.25 per hour bin), the difference was not statistically significant (generalized linear model, $F_{1,23}$ = 2.79, P = 0.10) (Fig. 5.7). Hence, diapausing females are as active as non-diapausing females.

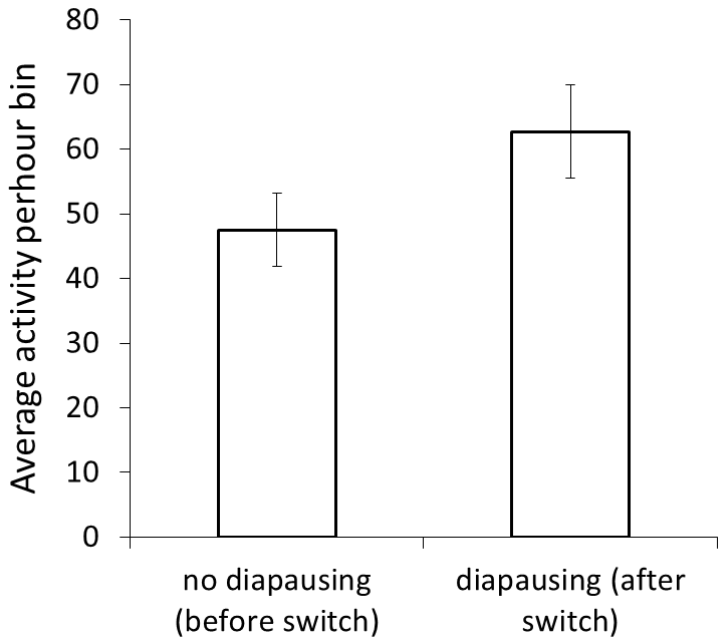

Figure 5.7 Level of activity during the light phase for *Nasonia vitripennis* females from the selected northern isofemale line under light:dark 12:12 before and after the switch point for diapause induction.

DISCUSSION

In this chapter we described natural variation in aspects of circadian locomotor activity in *Nasonia vitripennis* including the proportion of rhythmic individuals, a latitudinal cline in free running period and differences in level of activity between northern and southern isofemale lines. We observed significant variation in the proportion of rhythmic individuals and *tau* between females and males: males are on average more rhythmic than females and have shorter *tau* compared to females. Similar differences between sexes were observed previously in the laboratory strain *Nasonia* AsymC (Bertossa *et al.*, 2013). The difference in rhythmicity between males and females appears more evident in mated individuals. In fact, virgin individuals of both sexes are highly rhythmic in constant darkness and mated males maintain a high level of rhythmicity (although lower compared to virgin males), whereas females seem to lose their internal rhythmicity after mating. The loss of rhythmicity in mated females could be explained in relation to their life cycle. After mating, females disperse in search for new hosts to be parasitized, thus the necessity to move and explore different patches for laying the eggs might overcome the internal rhythmicity in locomotor activity and lead to arrhythmic activity. Similar effects of mating status on rhythmicity were found in queens of the ant species *Camponotus compressus* in which ovipositing queens showed arrhythmic locomotor activity during the egg laying phase and restored rhythmicity afterwards (Sharma et al., 2004). In addition, in our studies we found a significant interaction between location and mating status on proportion of rhythmic individuals, for

example the effect of mating status in females of the SWI population is larger than in the SCH population. This might be due to differences in the distribution of possible oviposition patches (e.g. nest boxes) in each sampling location. In a site where patches are close to each other, females do not need to search long for a new patch and might maintain their internal rhythmicity, whereas in a location where the patches are distant from each other, wasps need to move more before they can find a suitable oviposition site and consequently they might forgo their internal rhythmic cycle. Overall, the results show that the circadian systems controlling locomotor activity and possibly other behaviors are plastic and might be adjusted to different environmental conditions experienced during life to maximize the possibility of reproduction. It is possible that different environmental factors affect the rhythmic locomotor activity as was recently shown for the northern fly species *Drosophila montana*, where the proportion of rhythmic individuals was higher at lower temperature (Kauranen *et al.*, 2012). It would be interesting to investigate how environmental conditions (temperature and light intensity among others) will influence circadian locomotor activity behaviour of males and females in different species and test whether the level of plasticity will vary between populations from different geographical regions. Such studies could reveal differential selection pressures for stability of the circadian clock under different conditions.

We found a significant, albeit weak, latitudinal cline in the free running period for locomotor activity in virgin females with *tau* increasing towards higher latitudes. The variation in *tau* might derive from local adaptation to different environmental conditions, particularly the annual

progression in photoperiodic change that is strictly linked to latitude. It was hypothesized that free running periods longer than 24 hours might enhance the ability to respond to the light-on signal (Pittendrigh & Takamura, 1989) and therefore can be more adaptive at higher latitudes where a strong sensitivity to photoperiodic change is necessary for proper timing of the seasonal physiological responses (Michael *et al.*, 2003). The possible adaptive significance of clinal variation in *tau* is substantially unknown. The variation in free running period is likely the result of selection acting on traits genetically correlated with circadian rhythm, instead of acting on the circadian rhythm itself (Harano & Miyatake, 2010). In the case of *Nasonia vitripennis*, selection on photoperiodic diapause response might have led to the observed cline in tau, due to the shared genetic basis.

We investigated differences in level of activity between two selected northern and southern lines. Overall, higher level of activity was observed in the northern line compared to the southern line in constant light and during the light phase in a light:dark cycle 12:12. Such difference could result from variation in the sensitivity to light, for example northern individuals might be more sensitive to the light impulse and thus show a stronger response or it could be the result of adaptation to local light intensities in the wild. Testing variation in level of activity under different light intensities might clarify these hypotheses. Alternatively, the difference could reflect different properties of the circadian clock which allow specific daily patterns of activity in southern and northern populations. Such difference has been observed in several *Drosophila* species. In a study involving North American fly populations, species of

Drosophila from northern latitudes showed continuous activity during light phase, while southern species displayed bimodal activity with a resting phase during mid-day. There was a positive correlation between mid-day activity and latitudinal midpoint of the species range (Simunovic & Jaenike, 2006). Similar correlations were found in intraspecific studies of different populations of *D. melanogaster* (Allemand & David, 1976) and *D. ananassae* (Joshi, 1999). The lack of bimodal activity during light phase was also observed in the Northern species *D. montana* (Kauranen *et al.*, 2012). Although we did not observe a specific pattern of daily activity during the light phase in our selected southern line, it is possible that the resting periods during the daily light are present also in *Nasonia*, which would result in an overall lower level of activity during light phase in the southern line compared to northern line. This could be adaptive as it allows southern individuals to rest during the hot hours of summer mid-days and concentrate their activity during early morning and late evening. On the other hand, at higher latitude the continuous activity during the light phase is adaptive as the mild temperatures allow movement and activity during the whole day and thus maximal exploitation of resources during light phase. In addition, northern latitudes are characterized by short favourable seasons and therefore, individuals with a continuous activity during the light phase are favoured as they can maximize the use of resources which will be available only for a short period of time. Furthermore, the selected 12:12 light:dark cycle is a strong diapause inducing photoperiod for the northern line and females under this condition lay eggs that will develop into diapausing larvae and overwinter. It is possible that the higher level of activity is linked to physiological

changes in the females after exposure to short photoperiods which induce diapause production. In line with this hypothesis, our last experiment shows that females after the switch point tend to be more active than females before the switch point, although the difference was not statistically significant.

Another interesting observation was the higher level of activity observed in both northern and southern females that received a host for 2 hours every other day. These females showed high activity levels also during the dark phase. The level of activity was still very high when the females were continuously exposed to a host. Wasps can eat from the pupal host and can lay eggs inside it. Food availability and possibility to oviposit might represent strong stimuli for *Nasonia* wasps and their effect might mask the effect of light on circadian rhythmic behaviour. Notably, despite the increase of activity due to the presence of the host, the difference between northern and southern lines was maintained.

Overall the results described in this chapter show natural variation in some properties of the circadian clock and sensitivity to light that likely reflect different selection pressures in different locations. Interestingly, many traits related to circadian activity show a high level of plasticity which allows flexibility in daily activities depending on internal conditions (e.g. mating status) or external environmental conditions (e.g. light:dark cycle, presence of food). However, variation between geographic locations is maintained even in the plastic response to different stimuli. This suggests that natural selection acts on the response of the circadian system to the environment and not on the circadian clock *per se*. This is similar to the natural variation described for photoperiodic diapause

induction in *Nasonia* which is based on the differential response to light:dark cycle (chapter 2 of this thesis; Paolucci et al., 2013). These similarities further indicate that the circadian system and photoperiodism might be based on the same threshold mechanism, possibly involving the clock gene *period*, which allows proper responses to environmental stimuli.

Chapter 6

Summarizing discussion

Silvia Paolucci

***The genetic basis of adaptive variation in photoperiodic diapause induction in* Nasonia vitripennis**

Like the majority of insect species living in temperate zones, the parasitic wasp *Nasonia* survives the winter in diapause, a physiological state of dormancy induced by environmental cues such as photoperiod and temperature. In *Nasonia*, diapause occurs at the fourth larval instar and is induced by the adult mother that represents the sensitive stage. The adult female is able to detect the environmental cues and can direct the development of her offspring towards diapause or non-diapause larvae depending on the perceived stimulus. Photoperiod (length of light phase within the 24 hours period) is the most important cue for diapause induction in *Nasonia*, which is considered a long-day insect, i.e. short photoperiods induce and long photoperiods inhibit diapause. Saunders (2002) proposed that the mechanism of photoperiodic response consists of two components: a photoperiodic timer which measures the length of the day (or night), and a photoperiodic counter which counts the number of cycles. The counter uses the information from the timer to elicit the response after a threshold number of inductive photoperiodic cycles has been reached (Saunders, 2002). In *Nasonia*, the female exposed to short photoperiods will produce normal developing larvae at the beginning of her life and switch to the production of diapausing larvae after a threshold number of days (Saunders, 1966). Such threshold number is called *switch point* and is the expression of the photoperiodic counter. *Nasonia vitripennis* is widely distributed in areas covering a great diversity of environments. Diapause response of different populations is expected to match the seasonal environments of different geographic locations as a

result of local adaptation. The investigation of the genetic basis of such adaptive variation has been the focus of my thesis.

We described different aspects of photoperiodic diapause that vary across geographic clines according to latitude, with particular focus on the switch point for diapause induction, i.e. the expression of the counter component of the photoperiodic clock. We showed that clinal variation in photoperiodic diapause induction in *Nasonia vitripennis* is based on differences in the maternal sensitive phase. Southern populations, with a late switch point, show low proportions of diapause as the adult female produces non-diapausing offspring for a large part of her life before the switch and few diapausing offspring only after the late switch. Northern populations, with an early switch point, show high proportions of diapause offspring as the mother had time to produce many diapausing broods after the switch that occurred early in life. We proposed that natural selection acts on the threshold mechanism allowing an adaptive diapause response at each geographical location. No lines were found that lack the physiological capability to enter diapause or would enter obligate diapause (sampling covered a latitudinal range of 23 degrees), confirming that the observed variation is not based on a fixed developmental diapause pattern, but on the variation in sensitivity to photoperiod. Latitudinal variation in diapause response might result from selection on voltinism, i.e. the number of generations per year. Typically, northern populations are exposed to short growing seasons and they have univoltine or bivoltine life cycles. The number of generations during the breeding season increases towards the south where summers are longer and environmental conditions favourable for reproduction and growth

persist for several months. Field experiments should be performed to investigate voltinism in *N. vitripennis* at different latitudes. These experiments are necessary to understand the adaptive value of variation in switch point for diapause induction in regulating voltinism.

Our results showed that the observed variation in photoperiodic response is genetically determined, confirmed by the fact that phenotypic differences were observed between populations under the same photoperiodic condition (common garden type of experiments) and by the maintenance of such differences between lines after several generations of lab culturing. As a next step, we addressed the genetic basis of adaptive variation in photoperiodic response through a combination of QTL analysis and candidate gene approach. Given the results of the above common garden experiments, the analysis focused on the switch point, as main target of natural selection. By choosing two isofemale lines from the extremes of the cline and an intermediate photoperiod (light:dark 14:10) to score the diapause induction, clear phenotypic differences between the two lines could be observed. The reciprocal inter-line crosses yielded F1 females with 50% southern and 50% northern genome which showed an intermediate diapause response compared to the pure lines, suggesting either a polygenic basis for diapause induction with additive effects or a more simple inheritance with few factors and some degree of codominance. Additionally, the F1 females that originated from the two reciprocal crosses had different cytoplasm and similar nuclear genomic composition, which allowed to measure the effects of cytoplasmic components on diapause. These two groups of females showed similar response indicating that variation in switch point is not determined by

cytoplasmic factors but mainly by maternal nuclear genes. However, upon mating these females with males from the two parental lines, differences in diapause response were observed, indicating that interaction between the maternal and offspring genome or paternal factors are also affecting the diapauses response. Overall, our results confirm those of Saunder's that diapause regulation in *Nasonia* is largely governed by the female's nuclear genome (Saunders, 1965). However, other genetic and environmental factors may have a modulating effect. Future research could be directed to establishing whether the relative contributions of these genetic and environmental effects differ between geographical locations. This would provide more insight into how fine-scale variation in diapause as an adaptive trait can come about. For example, in the ground cricket *Allonemobius socius*, the relative importance of environmental factors for diapause induction increases in populations from southern locations leading to a higher plastic response compared to northern populations (Winterhalter & Mousseau, 2007). Such cline in the level of plasticity allows to fine-tune the diapause response to variable environmental conditions.

The QTL analysis revealed two large genomic regions involved in the switch point for diapause induction, one on chromosome 1 and one on chromosome 5, affecting the trait in an additive manner with partial dominance of northern alleles over southern alleles. This suggests that photoperiodic diapause induction has a simple genetic basis and is regulated by few loci. Although this should be confirmed with a more fine-scale QTL mapping, using a larger number of molecular markers, it is interesting that addition of SNP markers in the candidate locus

period/cycle increased the proportion of variance explained indicating that this locus has a major role in shaping diapause response. The significant association between *period/cycle* and the quantitative variation in photoperiodic diapause induction argues in favour of the functional involvement of the circadian clock in seasonal photoperiodic response which is the subject of a long-standing and still open debate.

The link between clock genes and diapause was further investigated by studying how allelic variation of *period* correlates with phenotypic variation in diapause. We sequenced DNA fragments in 12 exons of the *period* gene in the two selected northern and southern lines and we found three different haplotypes associated with different switch points, which was confirmed by inter- and intra-line crosses. Allelic *period* variation was also assessed for individuals from field lines previously scored for diapause response. Two main haplotypes were identified (based on two focal non-synonymous SNPs in exon 3 and 16, respectively) which showed a latitudinal cline in frequency, correlated to the latitudinal cline in switch point. We conclude from these results that the clock gene *period* has a role in adaptive variation of photoperiodic diapause induction in *N. vitripennis* and is likely involved in the photoperiodic counter, measured as switch point. Obviously, this does not exclude the involvement of other genes in photoperiodic response, as evident from the large genomic regions found in the QTL analysis. To identify these genes and possible interactions between them, a fine scale screening of the detected QTL regions should be performed. The relative role of each gene in diapause could be studied with introgression experiments in which different alleles are introgressed in a common genetic background and

subsequently associated with variation in phenotypic response.

Considerations on the role of* period *in diapause and link between circadian clock and photoperiodism

The details on the effect of different *period* alleles on the photoperiodic counter are not known and functional studies are required to unravel the exact role of *period* on adaptive variation of diapause. In this research, we characterized two main alleles coding for different forms of PER protein (two amino acid polymorphisms) with possibly variable functionality. However, the effect of the different *period* alleles might also reside in differential gene expression or even a combination of differential expression level and variation in protein functionality. In any case, *period* alleles appear to modify the switch point, defined as the required number of light:dark cycles for diapause induction. We do not know the proximate mechanism through which different *per* alleles affect the switch point. It is possible that under a certain photoperiodic condition, every light:dark cycle perceived by the organism activates an internal mechanism that leads to the production of a specific “diapause factor” which accumulates day by day. When the amount of such factor reaches a given threshold, a cascade of physiological events involving the production of neuro- and developmental hormones, is activated and leads to diapause induction. In the case of *Nasonia*, these events occur over two generations as the adult female perceives and interprets the photoperiodic environmental variation and transmits this through her eggs that then induce diapause in the larval offspring. The “diapause factor” can be any substance like a protein or a hormone which directly influences the output. It can also

involve additional genes and gene products that affect the trigger of the output. Several levels of complexity and interactions might be present between the perception of the light input and the signal that is transmitted to the egg.

Tagaya et al. (2010) proposed a model to explain how different populations can show variable diapause response under different photoperiods and how the variation in properties of the photoperiodic counter leads to variation in the critical photoperiods (Tagaya et al 2010; Goto 2013), thus confirming the action of natural selection on the counter component of the photoperiodic clock, hypothesized in the discussion of chapter 2 of this thesis. The Tagaya *et al.* model is a clear representation of the interrelationship between the photoperiodic timer that measures length of the day and the counter that counts and accumulates the number of inductive cycles. Based on this model, here I propose two alternatives in which *period* alleles can function within the seasonal photoperiodic response. For simplicity, I consider only two *per* alleles: per_S (South) and per_N (North) associated with late and early switch points, respectively. As in Tagaya et al. (2010), the model assumes an accumulation of light:dark cycle information and a threshold mechanism. In the first scenario (Fig. 6.1A) there is a fixed threshold level of the diapause factor required to trigger the output response and the two *per* haplotypes differently affect the light dependent accumulation rate of this factor in such a way that individuals carrying per_S accumulate less of this factor per day than that individuals carrying per_N and thus need more cycles to reach the threshold. This difference in accumulation rate can be based on different gene expression of per_S and per_N alleles which leads to

different level of the two corresponding protein forms. Alternatively, the difference can reside in the structure of the two proteins encoded by per_S and per_N alleles which could, for example, lead to different capacity to bind to other proteins and form functional complexes which in turn affects the production of the diapause factor. In the second scenario (Figure 6.1B), the *per* alleles might be associated with two different threshold levels: per_S individuals have a higher threshold and thus more cycles are required to reach it compared to the low threshold level associated with per_N which then requires fewer cycles. Obviously, a combination of the two scenarios is also possible, making the investigation of the specific function and regulation of *period* even more complicated.

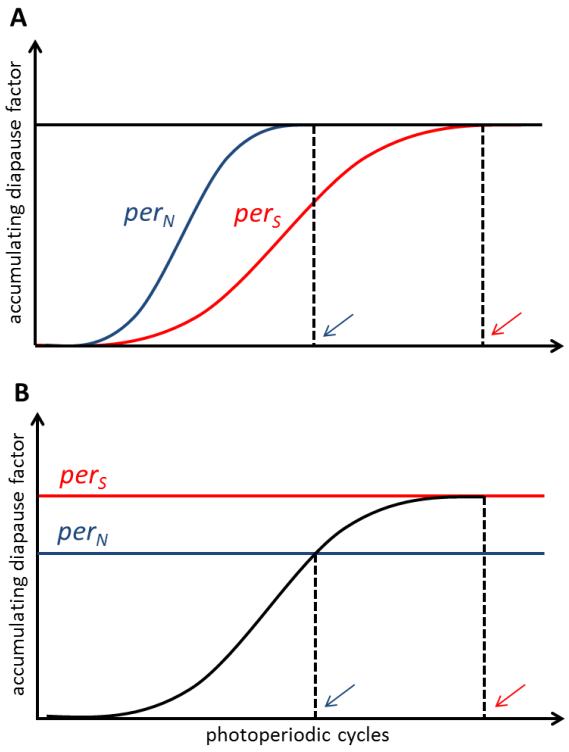

Figure 6.1 Two mechanisms proposed for the role of *period* in the threshold mechanism for photoperiodic response. **A)** Fixed threshold and two different accumulation rates determined by the two *period* variants. **B)** Fixed accumulation rate and two threshold levels determined by the two *period* variants. Blue arrows indicate a northern early switch point and red arrows indicate a southern late switch point.

Our findings about the involvement of *period* in variation of the threshold mechanism for diapause induction, unavoidably lead to the discussion about the functional link between the circadian clock and seasonal photoperiodic response. The long-standing debate on this topic is still open and vivid (Saunders, 2009; Schiesari et al, 2011; Meuti & Denlinger, 2013; Koštál, 2011; Hut & Beersma, 2011; Goto, 2013). Despite the current knowledge on the molecular basis of the circadian clock system in model species and recent studies on the association between clock genes and photoperiodic diapause, a consensus on the exact link between the circadian clock and photoperiodism has not been reached. In his review, Koštál (2011) proposes three possible interrelationships: A. independence – the two systems are physically separated and work independently from each other; B. cooperation – the two systems are physically separated but work in cooperation; C. unity – there is only one system with dual function. Studies on different species using variable approaches led to different conclusions with some support for all three hypotheses, suggesting that the nature and strength of the link between the circadian clock and photoperiodism might vary between species or even populations (Koštál 2011). Moreover, the link might depend on the characteristics of the circadian clock which is typically explained by either an internal coincidence model or an external coincidence model (Hut & Beersma 2011; Goto 2013). These two main types of the circadian clock might function differently with respect to the photoperiodic response (Saunders, 2009; Hut & Beersma 2011).

Given its adaptive value in regulating the seasonal response in a large variety of species and a multitude of environments, it does not come

as a surprise that photoperiodism is regulated at many different levels, each of which can affect the final response in a different way in a given species, in a given location and time of the year. Natural selection might have "used" various routes to shape the seasonal clock in response to different needs, resulting in a large range of possible mechanisms leading to adaptive response. Therefore, the search for a unique, comprehensive explanation of how photoperiodic response occurs in nature might still be very long and possibly not leading to a conclusive universal answer. An example of how natural selection could use alternative routes leading to similar phenotypes is the evolution of sex determination systems: studies in various taxa revealed great variation in mechanisms leading to the occurrence of only two sexes without exception (Beukeboom & Perrin, *in press*). The complexity of photoperiodism as an adaptive trait and the role of clock genes in diapause response in insects were the focus of the review/opinion paper by Emerson *et al* (2009). The authors suggest that the role of clock genes in photoperiodic diapause, if there is any, should be seen as gene pleiotropy or modular pleiotropy. In gene pleiotropy, a mutation or allelic variation at a clock gene can independently modify both the photoperiodic diapause induction and the circadian clock. In the modular pleiotropy model, the clock gene modifies the circadian clock mechanism as a module, which in turn modifies the photoperiodic response (Emerson *et al* 2009) (Fig. 6.2). In *Nasonia vitripennis*, sequence variation in the *period* gene affects the switch point for diapause induction. The two alternative mechanisms that I proposed (Fig 6.1) on how such effect is expressed can fit both the gene pleiotropy and modular pleiotropy model as they are not strictly linked to the types of relationship

between circadian clock and diapause. If *period* acts as isolated gene with pleiotropic effects on both the circadian clock and photoperiodic response, the different *period* alleles affecting diapause response may or may not affect the circadian clock. On the other hand, if *period* alleles modify the photoperiodic response through the modification of the circadian clock, then the effect of different *per* alleles will necessarily have to be observed in the circadian clock as well. In other words, showing that different *period* alleles affect both the circadian clock and photoperiodic diapause will still leave open the two possibilities of gene pleiotropy and modular pleiotropy, while showing that different *period* alleles only affect diapause but not the circadian clock will at least exclude the modular pleiotropy model.

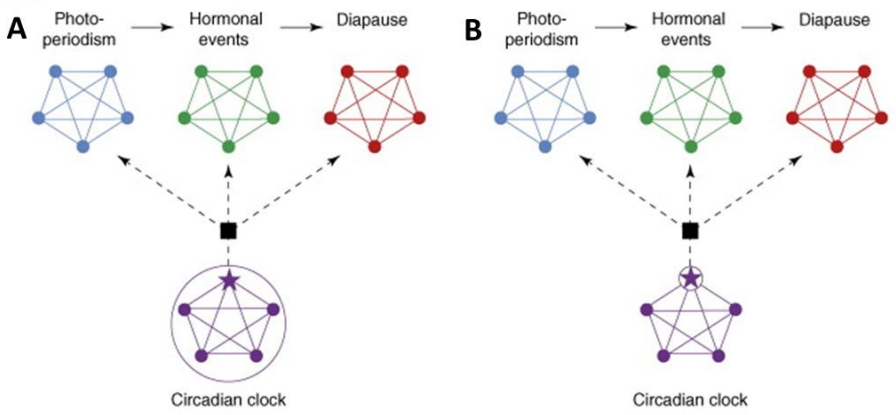

Figure 6.2 Effect of clock genes on diapause. **A)** Modular pleiotropy. **B)** Gene pleiotropy. The focus clock gene is represented with a star. The module is represented as a group of interacting genes (small filled circles) connected in a network (connecting lines forming pentagons). The black box represents the "unknown mechanism" through which clock genes might affect diapause (Emerson *et al*., 2009). I propose interpretations of this black box in the text.

We attempted to gain more insight in the relationship between both the circadian and seasonal clock by studying variation in some properties of the circadian clock in natural populations which show a latitudinal cline in diapause response and correlated latitudinal variation in *per* haplotype frequency. We studied locomotor activity rhythm under constant conditions and found variation in free running period (*tau*) between and within locations as well as a weak positive correlation between *tau* and latitude for virgin females only. These results do not allow to draw firm conclusions about a latitudinal cline in free running period. Further investigation of different aspects of the circadian clock is necessary to fully characterize variation in circadian rhythmicity along geographic gradients. Interestingly, we observed a consistent difference in the level of activity between two investigated northern and southern lines (which possess different switch point for diapause induction and different *per* haplotypes), with the northern line being more active than the southern line during light phase (almost no activity was registered during dark phase in absence of extra stimuli, e.g. host pupa). It is possible that such differences derive from variation in properties of the circadian clock which leads to different responses to light in the two lines perhaps due to different sensitivity to light intensity. The *period* alleles might be responsible for these differences. For example, the high activity level conferred by the per_N allele could reflect a highly active circadian clock and prompt response to light. Such a high cyclic activity allows regular large production of stimuli (mentioned above as “diapause factor”) that trigger diapause induction after a specific threshold. The low activity of per_S allele could reflect a more moderate activity of the circadian clock leading to

slower production of the diapause factor. This explanation would fit in the modular pleiotropy model, as the *per* alleles affect the efficiency of the circadian clock to produce the diapause factor which in turn affect diapause response. In this perspective, the photoperiodic response mechanism makes use of the circadian clock to give the stimulus for the production of "diapause factor" and the two will then be considered functionally linked. To verify these hypotheses, it would be useful to screen the *period* alleles in individuals showing different level of activity also from geographic locations and test the correlation between *period* alleles, diapause induction and circadian rhythmic activity.

Conclusion and future directions

The research presented in this thesis contributes to the understanding of the genetic basis of seasonal adaptation. It describes the natural variation in photoperiodic induction of diapause and its association with the circadian clock gene *period* in the parasitic wasp *Nasonia vitripennis*. The results give fuel to many lines of future research. As for the genetic basis of photoperiodic diapause, genome wide association mapping and QTL analysis with large numbers of molecular markers could be employed to identify more genes involved in seasonal response and their relative effects. Introgression experiments where different forms of specific genes are introgressed in standard genomic background can help to confirm their role in adaptive variation. For example, introgressing different *period* haplotypes in a common genetic background and studying corresponding phenotypic response will shed light on the details of the involvement of this gene in adaptive variation of diapause response.

At the mechanistic level, testing the proposed models for the involvement of *period* in the threshold mechanism regulating photoperiodic response will necessitate more knowledge on the physiological pathway leading to diapause. This requires to identify the "diapause factor", its target and how it is regulated. Such Information could be gained through gene expression studies combined with functional analysis of proteins encoded by the genes involved in the pathway. Lastly, detailed knowledge of the molecular basis of the circadian clock mechanism in *Nasonia vitripennis* will be useful to investigate the link between this clock and photoperiodism. The study of the molecular details of the *Nasonia* clock will benefit from the characterization of the clocks of closely related hymenopteran species. Recently, the molecular bases of the circadian clock in hymenopteran model species such as the honey bee *Apis mellifera* (Rubin *et al.*, 2006) and the ant *Solenopsis* (Ingram *et al.*, 2012) were characterized. These clocks appear to be different from the one of *Drosophila melanogaster* in the involvement and function of specific clock genes (Tomioka & Matsumoto, 2010). Nevertheless, the core mechanism based on a series of positive and negative feedback loops seems to be conserved. Very recently, Bertossa *et al*. (2014) showed the oscillating expression of the two key clock genes *period* and *cryptochrome* in *Nasonia* under different photoperiods. These genes are virtually synchronous in their expression and are set by light-on signal, similar to other hymenopterans, in which the clock relies on a functional dimer between PER and CRY proteins rather than PER and TIM (encoded by the *timeless* gene) as is the case in *Drosophila* (Yuan *et al.*, 2007). Modern sequencing technologies and availability of genomic tools will surely help identifying

other clock genes and their expression under specific photoperiodic regimes.

Last, but not least, it is important to stress that *Nasonia* proved to be an excellent organism for the study of seasonal adaptation, photoperiodic diapause induction and circadian rhythmicity. The clear photoperiodic response with very distinct sensitive and response stages allows to investigate various aspects of diapause separately and to focus on specific components of the response. Additionally, the maternal effect in diapause induction in *Nasonia* offers the opportunity to investigate general mechanisms of transgenerational signals and how parents can control the developmental fate of their offspring. The widespread distribution of *Nasonia vitripennis* with populations encountering a variety of seasonal environments provides the opportunity to analyse the adaptive variation of diapause and its genetic basis. All these factors combined with the increasing availability of genomic tools make *Nasonia* a powerful new model system for the study of genetics of adaptation to seasonality at all levels.

Appendices

Appendix 1

Appendix 1.1 Multiplex sets of primers for amplifying microsatellite markers used for the initial screening of the two selected northern and southern isofemale lines

Markers	Chr	Primer sequences (5' --> 3')	
SET 1		**Forward**	**Reverse**
Nv_107	3	CAGTCGGCAACAACAGATTG	CGATTCGATGAATGAGCAGA
Nv_200	1	TCCGCACGGTCAGTCTTT	CGGCGAATTTCGTTCTTC
Nv_26	4	CAGCTTTCCTTTGCTGCATATAG	CTCCTAGAGCTAGTATGAACCGTTAA
Nv_112	4	GCTCCTCCTTGTTTGCGTTA	TCGAGCGACGAGTGATATCTT
Nv_319	3	TTTGAGGTTATGCGTCGTTTC	GAGCGGAGTGCTTCATTCAG
Nv_309	4	TTCAGCTTCACGCTCAGGTA	GCGAGAGCAATCAGAAACAA
Nv_229	5	AAATATTGGCGCGGCAAC	CCAACAATGAGTGTATCCTAGGC
Nv_217	3	AATGGCATTATGCGAATGA	CTGCTCTCTGCATGAATCTTT
SET 2			
Nv_323	4	CTCGATTGCGAAACTGTTCA	TTTATACAGCGCAGGCACTAGA
Nv_209	2	CCAACTTCTTATTCGTAAGGGAA	ACCATTCGCTGGCTGGTA
Nv_210	2	AGGACGCAGCTAGGTGGC	CCTCGTCGATCAAGAGGC
Nv_212	3	CATAAATACATTTGGGTCTCCC	TGGAGTCCAGCTAGGATTCTAA
Nv_325	5	AGCGCAAAATGTTCAGCTC	ATTAAGAAGGCCCCCGAAG
Nv_203	1	CGTGCACTTTCTCTCCCTTT	TGCACATTCGCGAAACAC
Nv_219	4	GCCTGCCGTACAATCAAA	GAAACGCGACGCTGTTAG

Markers	Chr	Primer sequences (5' --> 3')	
SET 3		**Forward**	**Reverse**
Nv_302	3	TCTTTGGTCGATCGATTTTTC	TTCGTTGTGACCAAGCAGTT
Nv_303	3	GACAATAGCCGCTACGGAAA	CGTCGTTCTGCTGCTTGTC
Nv_311	1	ACTGGCGAAAGCTCAAACC	TCGAGCTTTGTTCTGGGATA
Nv_320	2	ATGTGCTGGCGAAGAAAAAT	ACGTTTCTGCTGCTGCTTCT
Nv_204	1	GCGCGTCGTCTTGTTTAA	TTACCCGGCCGATGTTAG
Nv_118	4	AGAATCGAAGCGGGATTAGC	TTAAATCCCAGCCAGACGAG
Nv_220	4	CGCTACTCTCGTCAACCTGTAA	GTTCGCTCGTCGATGATAA
SET 4			
Nv_41	3	GTCAGACGTGGGCTTTGTC	TTATGCGCCACACACACC
Nv_321	4	CGGTGAGACTCGTGAGATGA	AACCGCAGCTCTCAACATTT
Nv_322	5	CGAAAGAAGCCAAGCATAGAA	GAGAAAAATCGGGTCGAAGT
Nv_306	2	TGCTCGGATTTCGAACATTT	GCGGATGTTGTTCCGTTATT
Nv_308	1	ATTCGGAATCCACGAAACG	TAGGGCGCGTATAGATCGAG
Nv_205	1	GCTCGAGGAGGCCATATC	CCTCGATAGCTGGCAACC
Nv_312	3	GCACACACTCGCGATAAGAA	TGTAGAATTCGCCGTGTGAC
SET 5			
Nv_206	2	CGATGTTGCGACCGTCTATA	TCCGATCAAATCGAATTACTGTA
Nv_218	4	TCGCTTAGATAATTGCCAGAC	ACAGATATACTCTCGTGCAGGAG
Nv_226	5	GTGACGCTTCGCAGCTGT	GGATATTCGCGGCGAGTG
Nv_301	2	CGAGGCAACGATTTTCTTTC	CGTATCGCACTGCTTGTGTT
Nv_313	5	GAAGCTGCGGGTTAAGTGTG	CGCTACTTTATGCCAGTTACGG
Nv_46	5	TTACGTCAAGGTATAGCTGC	GAATAAGTGGCTGAAAGTTCC

Appendix 1.2 Multiplex sets of primers for amplifying microsatellite markers selected for QTL analysis

Markers	Chr	Primer sequences (5' --> 3')	
SET 1		**Forward**	**Reverse**
Nv_218	4	TCGCTTAGATAATTGCCAGAC	ACAGATATACTCTCGTGCAGGAG
Nv_26	4	CAGCTTTCCTTTGCTGCATATAG	CTCCTAGAGCTAGTATGAACCGTTAA
Nv_319	3	TTTGAGGTTATGCGTCGTTTC	GAGCGGAGTGCTTCATTCAG
Nv_229	5	AAATATTGGCGCGGCAAC	CCAACAATGAGTGTATCCTAGGC
Nv_41	3	GTCAGACGTGGGCTTTGTC	TTATGCGCCACACACACC
SET 2			
Nv_220	4	CGCTACTCTCGTCAACCTGTAA	GTTCGCTCGTCGATGATAA
Nv_322	5	CGAAAGAAGCCAAGCATAGAA	GAGAAAAATCGGGTCGAAGT
Nv_118	4	AGAATCGAAGCGGGATTAGC	TTAAATCCCAGCCAGACGAG
Nv_320	2	ATGTGCTGGCGAAGAAAAAT	ACGTTTCTGCTGCTGCTTCT
Nv_303	3	GACAATAGCCGCTACGGAAA	CGTCGTTCTGCTGCTTGTC
SET 3			
Nv_205	1	GCTCGAGGAGGCCATATC	CCTCGATAGCTGGCAACC
*Nv_206	2	CGATGTTGCGACCGTCTATA	TCCGATCAAATCGAATTACTGTA
Nv_226	5	GTGACGCTTCGCAGCTGT	GGATATTCGCGGCGAGTG
Nv_46	5	TTACGTCAAGGTATAGCTGC	GAATAAGTGGCTGAAAGTTCC
Nv_313	5	GAAGCTGCGGGTTAAGTGTG	CGCTACTTTATGCCAGTTACGG
SET 4			
Nv_107	3	CAGTCGGCAACAACAGATTG	CGATTCGATGAATGAGCAGA
Nv_112	4	GCTCCTCCTTGTTTGCGTTA	TCGAGCGACGAGTGATATCTT
Nv_200	1	TCCGCACGGTCAGTCTTT	CGGCGAATTTCGTTCTTC
Nv_301	2	CGAGGCAACGATTTTCTTTC	CGTATCGCACTGCTTGTGTT
Nv_308	1	ATTCGGAATCCACGAAACG	TAGGGCGCGTATAGATCGAG
SET 5			
*Nv_203	1	CGTGCACTTTCTCTCCCTTT	TGCACATTCGCGAAACAC
Nv_204	1	GCGCGTCGTCTTGTTTAA	TTACCCGGCCGATGTTAG
Nv_210	2	AGGACGCAGCTAGGTGGC	CCTCGTCGATCAAGAGGC
Nv_302	3	TCTTTGGTCGATCGATTTTTC	TTCGTTGTGACCAAGCAGTT
Nv_306	2	TGCTCGGATTTCGAACATTT	GCGGATGTTGTTCCGTTATT

*not used in the QTL analysis

Appendix 2

Primers for diagnostic SNP markers in candidate genes

name	**Forward 5'→ 3'**	**Reverse 5'→ 3'**
Nv_*per*1	CCAACATCAGGAAGGAGGTG	TGTCGGTGACTTGGTACGAG
Nv_*cycle*1	GTTCGAATCGCCAGAATGTT	ATCACGCCGTTGAATTGATA
Nv_*cry*1	GCATGAAGGTGAGCGTTTTT	GCAGAAGCTCGTCGAATACC

Pcr profile: 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C and 45 s at 72 °C, followed by 7 min at 72 °C

Appendix 3

Sequencing protocol

PCR amplified products were purified from excess of primers, dNTPs and Taq polymerase. A 4 µl reaction mix composed of 0.08 µl of ExoI (Exonuclease I, 20U/µl), 0.12 µl of FAP (FastAP thermosensitive alkaline phosphasate, 1U/µl) and 3.8 µl of sterile water was added to 5µl of each PCR product. The enzymatic reaction was activated for 22 min at 37°C for 22 min followed by 15 min at 80°C. The cleaned PCR products were mixed with primers, BigDye Termination mix, sequencing reaction buffer (company: Applied Biosystems) and water and the sequencing reaction was performed using the following steps: 1 min at 95°C followed 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. The sequencing reaction products were purified with Sephadex columns (Sephadex G50 Fine from Amersham Biosciences). The cleaned products were dried, re-dissolved in Hi-Di Formamide (Applied Biosystems) and Sanger-sequenced on a 3730 DNA Sequencer (Applied Biosystems). The chromatograms of each sequence were analysed with BioEdit Sequence alignment Editor (Hall, 1999) and the sequences were aligned using the software for molecular evolutionary genetic analysis MEGA 5 (Tamura *et al.*, 2011).

Appendix 4

Primers for amplifying exon regions of *period*

name	**Forward 5'→ 3'**	**Reverse 5'→ 3'**
Nv_*per*2	AGCTTCAGTTCACCTCGTCG	TATCGCCATCCTCCCAGATC
Nv_*per*3	GAATGTGTGAATGCGCAAAG	GGCATGTGAACATTTCGTTG
Nv_*per*4	GCAAATATTGACGCGCAG	GTCGAGATGTAATTACCTGGC
Nv_*per*5	CGAATGCACACTTTCAGCAC	GCTTTTGTCAGTCTGGTCGG
Nv_*per*6	ATAACGTTGCCGGCGGCGAA	TGGGTACGGAGAGCGGAGGC
Nv_*per*7	ATATTCGCAAAGGGATCACG	CCAAGATTCAACGAGCCAAT
Nv_*per*8	ATTGGCTCGTTGAATCTTGG	TGTGGAGATTCAGCAACGTC
Nv_*per*9	ACCAACCAGTGCCAACTTTC	AGCAGTTGCCAGAGATGGAT
Nv_*per*10	CCCGCAAATGTTACCAGTCT	CACAGAAGTTTCTGGCACGA
Nv_*per*11	CCTGCCCTGACCACTATGAT	AGGCAGTGTAGCGTCGATCT
Nv_*per*12	GGTGACTGCTCTTGCAGTTG	CAAAAACTTTGCGCATCTGA
Nv_*per*13	GAGTCAGTCAGATGCGCAAA	CTATTCGTACCGGCATCGTC

Primers for amplifying exon regions of *cycle*

name	**Forward 5'→ 3'**	**Reverse 5'→ 3'**
Nv_*cycle*2	ACCAACAGCAGCAGCAACTA	AAGACTCACTGGCAGAAGAGC
Nv_*cycle*3	TCCGATAGTCGTTCCAAAGC	TCGACGAAATATCGATGACG
Nv_*cycle*4	TGTTTGCGCGAATAGCTTTA	GGAACTTGCCGTCAATGG
Nv_*cycle*5	CCATTGACGGCAAGTTCC	TATCCTCTCGTGGCACACAC
Nv_*cycle*6	GTGTGTGCCACGAGAGGATA	TACTCGTTGCTCTGCTGTGC
Nv_*cycle*7	GTTACGCAGGTAACGGTGGT	CCGGACCATATCTTTCTCCA
Nv_*cycle*8	TTCAGAACTGCACGCTTGAT	TCAGTAGGGACAGGGTTTGG

PCR profile: 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C and 45 s at 72 °C, followed by 7 min at 72 °C

Bibliography

Reference list

Ahmed, U., Shi, Z., Guo, Y., Zou, X. & Hao, Z. 2007. Maternal photoperiod effect on and geographic variation of diapause incidence in *Cotesia plutellae* (Hymenoptera: Braconidae) from China. *Appl. Entomol.* 42: 383–389

Allemand, R. & David, J.R. 1976. The circadian rhythm of oviposition in *Drosophila melanogaster*: a genetic latitudinal cline in wild populations. *Experientia* 32: 1403–1405

Avila, F.W., Sirot, L.K., LaFlamme, B.A., Rubinstein, C.D. & Wolfner, M.F. 2011. Insect seminal fluid proteins: identification and function. *Annu. Rev. Entomol.* 56: 21–40

Bale, J.S. & Hayward, S.A. 2010. Insect overwintering in a climate change. *J. Exp. Biol.* 213: 980-994

Beach, R. 1978. The required day number and timely induction of diapause in geographic strains of the mosquito *Aedes atropalpus*. *J. Insect Physiol.* **24**: 449–455

Bean, D.W., Dalin P. & Dudley, T.L. 2012. Evolution of critical day length for diapause induction enables range expansion of *Diorhabda carinulata*, a biological control agent against tamarisk (*Tamarix* spp.). *Evol. Appl.* 5: 511–523

Bertossa, R.C., van Dijk, J., Diao, W., Saunders, D., Beukeboom, L.W. & Beersma, D.G.M. 2013. Circadian Rhythms Differ between Sexes and Closely Related Species of *Nasonia* Wasps. *PLoS One* 8: e60167

Bertossa, R.C., van de Zande, L., Beukeboom, L.W. & Beersma, D.G.M. 2014 Phylogeny and oscillating expression of *period* and *cryptochrome* in short and long photoperiods suggest a conserved function in *Nasonia vitripennis*. *Chronobiol Int.* Early online doi:10.3109/07420528.2014.880451

Beukeboom, L.W. & Desplan, C. 2003. Quick guide. *Nasonia*. *Curr. Biol.* 13: R860-R860

Beukeboom, L.W., Niehuis, O., Pannebakker, B.A., Koevoets, T., Gibson, J.D., Shuker, D.M., *et al.* 2010. A comparison of recombination frequencies in intraspecific versus interspecific mapping populations of *Nasonia*. *Heredity* 104: 302–309

Beukeboom, L.W. & Perrin, N. *in press. The evolution of sex determination*. Oxford University Press

Bradshaw, W.E. & Lounibos, L.P. 1977. Evolution of dormancy and its photoperiodic control in pitcher-plant mosquitoes. *Evolution* 31: 546-567

Bradshaw, W.E. & Holzapfel, C.M. 2001. Genetic shift in photoperiodic response correlated with global warming. *Proc. Nat. Acad. Sci. U.S.A*. 98: 14509–14511

Bradshaw, W.E. & Holzapfel, C.M. 2007. Evolution of Animal Photoperiodism. *Annu. Rev. Ecol. Evol. Syst.* 38: 1–25

Breeuwer, J.A. & Werren, J.H. 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* 346: 558–60

Broman, K.W. & Sen, S. 2009. *A Guide to QTL Mapping with R/qtl*. Springer, New York, USA

Bünning E. 1936. Die endogene Tagesrhythmik als Grundlage der photoperiodischen Reaktion. *Ber dtsch bot Ges.* 54: 590–607

Chen, C., Xia, Q.-W., Chen, Y.-S., Xiao, H.-J. & Xue, F.-S. 2012. Inheritance of photoperiodic control of pupal diapause in the cotton bollworm, *Helicoverpa armigera* (Hübner). *J. Insect Physiol.* 58: 1582–1588

Clark, A.G. 1990. Inference of haplotypes from PCR-amplified samples of diploid populations. *Mol. Biol. Evol.* 7: 111–22

Churchill, G.A. & Doerge, R.W. 1994. Empirical threshold values for quantitative trait mapping. *Genetics*, 138: 963–971

Costa, R., Peixoto, A.A., Barbujani, G. & Kyriacou, C.P. 1992. A latitudinal cline in a *Drosophila* clock gene. *Proc. Biol. Sci.* 250: 43–49

Danks, H. 1987. *Insect Dormancy: An Ecological Perspective*. Biological Survey of Canada, Ottawa

Danilevskii, A.S. 1965. *Photoperiodism and seasonal development of insects*. Oliver & Boyd Ltd, London, UK

Darling, D.C. & Werren, J.H. 1990. Biosystematics of *Nasonia* (Hymenoptera, Pteromalidae): two new species reared from birds' nests in North-America. *Ann. Entomol. Soc. A.* 83: 352–370

Demont, M. & Blanckenhorn, W.U. 2008. Genetic differentiation in diapause response along a latitudinal cline in European yellow dung fly populations. *Ecol. Entomol.* 33: 197–201

Demont, M., Blanckenhorn, W.U., Hosken, D.J. & Garner, T.W. 2008. Molecular and quantitative genetic differentiation across Europe in yellow dung flies. *J.Evol. Biol.* 21: 1492-1503

Emerson, K.J., Bradshaw, W.E. & Holzapfel, C.M. 2009. Complications of complexity: integrating environmental, genetic and hormonal control of insect diapause. *Trends Genet*. 25: 217-225

Fabian, D.K., Kapun, M., Nolte, V., Kofler, R., Schmidt, P.S., Schlötterer, C., *et al.* 2012. Genome-wide patterns of latitudinal differentiation among populations of *Drosophila melanogaster* from North America. *Mol. Ecol.* 21: 4748–4769

Gienapp, P., Teplitsky, C., Alho, J.S., Mills, J.A., & Merilä, J. 2008. Climate change and evolution: disentangling environmental and genetic responses. *Mol. Ecol*. 17: 167-178

Gomi, T. 1997. Geographic variation in critical photoperiod for diapause induction and its temperature dependence in *Hyphantria cunea* Drury (Lepidoptera:Arctiidae). *Oecologia* 111: 160–165.

Gomi, T. 2007. Seasonal adaptations of the fall webworm *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae) following its invasion of Japan. *Ecol. Res.* 22: 855–861

Goto, S.G. 2013. Roles of circadian clock genes in insect photoperiodism. *Entomol. Sci.* 16: 1–16

Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices. [2.9.3]

Grillenberger, B.K., Gadau, J., Bijlsma, R., Van de Zande, L. & Beukeboom, L.W. 2009. Female dispersal and isolation-by-distance of Nasonia vitripennis populations in a local mate competition context. *Entomol. Exp. Appl.* 132: 147-154

Grillenberger, B.K., Koevoets, T., Burton-Chellew, M.N., Sykes E.M., Shuker D.M., Van de Zande, L., Bijlsma, R., Gadau, J. & Beukeboom, L.W. 2008. Genetic structure of natural *Nasonia vitripennis* populations: validating assumptions of sex-ratio theory. *Mol. Ecol*. 17: 2854-2864

Hall, T.A. 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acid. S.* 41: 95-98

Han, B. & Denlinger, D.L. 2009. Length variation in a specific region of the period gene correlates with differences in pupal diapause incidence in the flesh fly, Sarcophaga bullata. *J. Insect Physiol.* 55: 415–418

Hardin, P.E. 2005. The circadian timekeeping system of Drosophila. *Curr. Biol.* 15: R714–R22

Hoffmann, A.A. & Sgrò, C.M. 2011. Climate change and evolutionary adaptation. *Nature* 470: 479-485

Huang, L.-L., Chen, C., Xiao, L., Xia, Q.-W., Hu, L.-T. & Xue, F. 2013. Geographical variation and inheritance of the photoperiodic response controlling larval diapause in two distinct voltine ecotypes of the Asian cornborer *Ostrinia furnacalis*. *Physiol. Entomol.* 38: 126–132

Hut, R.A. & Beersma, D.G.M. 2011. Evolution of time-keeping mechanisms: early emergence and adaptation to photoperiod. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 366: 2141–2154

Hut, R.A., Paolucci, S., Dor, R., Kyriacou, C.P. & Daan, S. 2013. Latitudinal clines: an evolutionary view on biological rhythms. *Proc. R. Soc. B Biol. Sci.* 280: :20130433

Ingram, K.K., Kutowoi, A., Wurm, Y., Shoemaker, D., Meier, R. & Bloch, G. 2012. The molecular clockwork of the fire ant *Solenopsis invicta*. *PLoS One* 7: e45715

Ikeno, T., Tanaka, S.I., Numata, H. & Goto, S.G. 2010. Photoperiodic diapause under the control of circadian clock genes in an insect. *BMC Biol.* 8: 116

Ikeno, T., Numata, H. & Goto, S.G. 2011. Circadian clock genes *period* and *cycle* regulate photoperiodic diapause in the bean bug *Riptortus pedestris* males. *J. Insect Physiol.* 57: 935–8

Joshi, D.S. 1999. Latitudinal variation in locomotor activity rhythm in adult *Drosophila ananassae*. *Can. J. Zool.* 77: 865–870

Kauranen, H., Menegazzi, P., Costa, R., Helfrich-Förster, C., Kankainen, A. & Hoikkala, A. 2012. Flies in the North: Locomotor Behavior and Clock Neuron Organization of *Drosophila montana*. *J. Biol. Rhythms* 27: 377–387

Koevoets, T., Niehuis, O., van de Zande, L. & Beukeboom, L.W. 2012. Hybrid incompatibilities in the parasitic wasp genus *Nasonia*: negative effects of hemizygosity and the identification of transmission ratio distortion loci. *Heredity.* 108: 302–11

Koštál, V. & Shimada, K. 2001. Malfunction of circadian clock in the non-photoperiodic-diapause mutants of the drosophilid fly, *Chymomyza costata*. *J. Insect Physiol.* 47: 1269–1274

Koštál, V. 2011. Insect photoperiodic calendar and circadian clock: independence, cooperation, or unity? *J. Insect Physiol.* 57: 538–56

Kurota, H. & Shimada, M. 2003. Geographical variation in photoperiodic induction of larval diapause in the bruchid beetle, *Bruchidius dorsalis*: polymorphism in overwintering stages. *Entomol. Exp. Appl.* 107: 11-18

Kyriacou, C.P., Peixoto, A. a, Sandrelli, F., Costa, R. & Tauber, E. 2008. Clines in clock genes: fine-tuning circadian rhythms to the environment. *Trends Genet.* 24: 124–32

Lankinen, P. 1986. Geographical variation in circadian eclosion rhythm and photoperiodic adult diapause in *Drosophila littoralis*. *J. Comp. Physiol. A* 159: 123–142

Lankinen, P. 1993. North-south differences in circadian eclosion rhythm in European populations of *Drosophila subobscura*. *Heredity.* 71: 210–218

Lee, S.F., Sgrò, C.M., Shirriffs, J., Wee, C.W., Rako, L., van Heerwaarden, B., *et al.* 2011. Polymorphism in the *couch potato* gene clines in eastern Australia but is not associated with ovarian dormancy in *Drosophila melanogaster*. *Mol. Ecol.* **20**: 2973–84

Leinonen, T., O'Hara, R.B., Cano, J.M. & Merilä, J. 2008. Comparative studies of quantitative trait and neutral marker divergence: a meta-analysis. *J. Evol. Biol.* 21: 1-17

Leisnham, P.T., Towler, L. & Juliano, S.A. 2011. Geographic variation of photoperiodic diapause but not adult survival or reproduction of the invasive mosquito *Aedes albopictus* (Diptera: Culicidae) in North America. *Ann. Entomol. Soc. Am.* 104: 1309-1318

Lewontin, R.C. 1964. The Interaction of Selection and Linkage. I. General Considerations; Heterotic Models. *Genetics* 49: 49–67

Librado, P. & Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451–1452

Maniatis., T., Fritsch, E.F. & Sambrook, J. 1982. *Molecular Cloning: A Laboratory Manual*, 11th ed. Cold Spring Harbor Laboratory Press, New York

Mathias, D., Jacky, L., Bradshaw, W.E. & Holzapfel, C.M. 2007. Quantitative trait loci associated with photoperiodic response and stage of diapause in the pitcher-plant mosquito, *Wyeomyia smithii*. *Genetics* **176**: 391–402

Mcwatters, H.G. & Saunders, D.S. 1996. The influence of each parent and geographic origin on larval diapause in the blow fly, *Calliphora vicina. J. Insect Physiol.* 42: 721–726

McWatters, H.G. & Saunders, D.S. 1998. Maternal temperature has different effects on the photoperiodic response and duration of larval diapause in blow fly (*Calliphora vicina*) strains collected at two latitudes. *Physiol. Entomol.* 369–375

Meuti, M.E. & Denlinger, D.L. 2013. Evolutionary links between circadian clocks and photoperiodic diapause in insects. *Integr. Comp. Biol.* 53: 131-143

Michael, T.P., Salomé, P. a, Yu, H.J., Spencer, T.R., Sharp, E.L., McPeek, M. a, *et al.* 2003. Enhanced fitness conferred by naturally occurring variation in the circadian clock. *Science* 302: 1049–1053

Mousseau, T. a & Dingle, H. 1991. Maternal Effects in Insect Life Histories. *Annu. Rev. Entomol.* 36: 511–534

Nei, M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York, NY, USA

Nelson, R.J., Denlinger, D.L. & Somers, D.E. 2010. *Photoperiodism. The Biological Calendar*. Oxford University Press, USA, New York

Niehuis, O., Gibson, J.D., Rosenberg, M.S., Pannebakker, B. A., Koevoets, T., Judson, A.K., *et al.* 2010 Recombination and its impact on the genome of the haplodiploid parasitoid wasp *Nasonia*. *PLoS One* 5: e8597

O'Malley, K.G., Ford, M.J. & Hard, J.J. 2010. *Clock* polymorphism in Pacific salmon: evidence for variable selection along a latitudinal gradient. *Proc. Biol. Sci.* 277: 3703–3714

Orr, H.A. 2005. The genetic theory of adaptation: a brief history. *Nat. Rev. Genet.* **6**: 119–27

Pannebakker, B.A., Niehuis, O., Hedley, A., Gadau, J. & Shuker, D.M. 2010. The distribution of microsatellites in the *Nasonia* parasitoid wasp genome. *Insect Mol. Biol.* 19(Suppl. 1): 91–98

Pannebakker, B.A., Watt, R., Knott, S. a, West, S. a & Shuker, D.M. 2011. The quantitative genetic basis of sex ratio variation in Nasonia vitripennis: a QTL study. *J. Evol. Biol.* 24: 12–22

Paolucci, S., van de Zande, L. & Beukeboom, L.W. 2013. Adaptive latitudinal cline of photoperiodic diapause induction in the parasitoid *Nasonia vitripennis* in Europe. *J. Evol. Biol.* 26: 705-718

Pegoraro, M., Noreen, S., Bhutani, S., Tsolou, A., Schmid, R., Kyriacou, C.P., *et al.* 2014. Molecular evolution of a pervasive natural amino-acid substitution in *Drosophila cryptochrome*. *PLoS One* **9**: e86483

Pittendrigh, C.S. & Takamura, T. 1989. Latitudinal clines in the properties of a circadian pacemaker. *J. Biol. Rhythms* 4: 217–235

Pultz, M.A. & Leaf, D.S. 2003. The jewel wasp *Nasonia*: querying the genome with haplo-diploid genetics. *Genesis* **35**: 185–91

R Development Core Team 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/

Reznik, S.Y, Kats, T.S., Umarova, T.Y. & Voinovich, N.D. 2002. Maternal age and endogenous variation in maternal influence on photoperiodic response in the progeny diapause in *Trichogramma embryophagum* (Hymenoptera: Trichogrammatidae). *Eur. J. Entomol.* 99: 175-179

Rubin, E.B., Shemesh, Y., Cohen, M., Elgavish, S., Robertson, H.M. & Bloch, G. 2006. Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock. *Genome Res.* 16: 1352-1365

Sadakiyo, S. & Ishihara, M. 2011. Rapid seasonal adaptation of an alien bruchid after introduction: geographic variation in life cycle synchronization and critical photoperiod for diapause induction. *Entomol. Exp. Appl.* 140: 69–76

Sarup, P., Frydenberg, J. & Loeschcke, V. 2009. Local adaptation of stress related traits in *Drosophila buzzatii* and *Drosophila simulans* in spite of high gene flow. *J. Evol. Biol.* 22: 1111-1122

Saunders, D.S. 1962. The effect of the age of female *Nasonia vitripennis* (Walker) (Hymenoptera, Pteromalidae) upon the incidence of larval diapause. *J. Insect Physiol.* 8: 309-318

Saunders, D.S. 1965. Larval diapause of maternal origin: Induction of diapause in *Nasonia vitripennis* (Walk.) (Hymenoptera: Pteromalidae). *J. Exp. Biol.* 42: 495-508

Saunders, D.S. 1966. Larval diapause of maternal origin II: The effect of photoperiod and temperaure on *Nasonia vitripennis J. Insect Physiol.* 12: 569-581

Saunders, D.S. 1966. Larval diapause of maternal origin III. The effect of host shortage on *Nasonia vitripennis*. *J. Insect Physiol.* 12: 899–908

Saunders, D.S. 1969. Diapause and photoperiodism in the parasitic wasp Nasonia vitripennis, with special reference to the nature of the photoperiodic clock. *Symp. Soc. Exp. Biol.* 23: 301–29

Saunders, D. 1973. Thermoperiodic control of diapause in an insect: theory of internal coincidence. *Science.* 181: 358-360

Saunders, D.S. 2002. *Insect Clocks*. 3ed ed. Elsevier, Amsterdam

Saunders, D.S. 2009. Circadian rhythms and the evolution of photoperiodic timing in insects. *Physiol. Entomol.* 34: 301–308

Saunders, D.S. & Bertossa, R.C. 2011. Deciphering time measurement: the role of circadian "clock" genes and formal experimentation in insect photoperiodism. *J. Insect Physiol.* 57: 557–566

Saunders, D.S., Sutton, D. & Jarvis, R.A. 1970. The effect of host species on diapause induction in Nasonia vitripennis. *J. Insect Physiol.* 16: 405–416

Sawyer, L.A., Hennessy, J.M., Peixoto, A.A., Rosato, E., Parkinson, H., Costa, R. & Kyriacou, C.P. 1997 Natural variation in a *Drosophila* clock gene and temperature compensation. *Science* 278: 2117– 2120

Sawyer, L., Sandrelli, F., Pasetto, C. & Peixoto, A. 2006. The *period* gene Thr-Gly polymorphism in Australian and African *Drosophila melanogaster* populations: implications for selection. *Genetics* 480: 465–480

Scharf, I., Bauerfeind, S.S., Blanckenhorn, W.U. & Schäfer, M.A. 2010. Effects of maternal and offspring conditions on growth, development and diapause in latitudinal yellow dung fly populations. *Clim. Res*. 43: 115-125

Schiesari, L., Kyriacou, C.P. & Costa, R. 2011. The hormonal and circadian basis for insect photoperiodic timing. *FEBS Lett.* 585: 1450-1460

Schmidt, P.S., Matzkin, L., Ippolito, M. & Eanes, W.F. 2005. Geographic variation in diapause incidence, life-history traits, and climatic adaptation in *Drosophila melanogaster*. Evolution 59: 1721-32

Schmidt, P.S., Zhu, C.-T., Das, J., Batavia, M., Yang, L. & Eanes, W.F. 2008. An amino acid polymorphism in the couch potato gene forms the basis for climatic adaptation in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 105: 16207–11

Sharma, V.K., Lone, S.R. & Goel, A. 2004. Clocks for sex: loss of circadian rhythms in ants after mating? *Naturwissenschaften* 91: 334–337

Shimizu, T. & Kawasaki, K. 2001. Geographic variability in diapause response of Japanese *Orius* species. *Entomol. Exp. Appl.* 98: 303–316

Simunovic, A. & Jaenike, J. 2006. Adaptive variation among drosophila species in their circadian rhythms. *Evol. Ecol. Res.* 8: 803–811

Sokolova, I. V. 2007. Univoltine seasonal cycle and obligate diapause in the noctuid moth *Charanyca trigrammica* Hufn. (Lepidoptera, Noctuidae). *Entomol. Rev.* 87: 793-798

Stehlík, J., Závodská, R., Shimada, K., Sauman, I. & Kostál, V. 2008. Photoperiodic induction of diapause requires regulated transcription of *timeless* in the larval brain of *Chymomyza costata*. *J. Biol. Rhythms* 23: 129–39

Tagaya, J., Numata, H. & Goto, S.G. 2010. Sexual difference in the photoperiodic induction of pupal diapause in the flesh fly *Sarcophaga similis*. *Entomol. Sci.* 13: 311–319

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731–2739

Tauber, M.J., Tauber, C.A. & Masaki, S. 1986. *Seasonal Adaptations of Insects*. Oxford University Press, USA

Tauber, E., Zordan, M., Sandrelli, F., Pegoraro, M., Osterwalder, N., Breda, C., *et al.* 2007. Natural selection favors a newly derived timeless allele in *Drosophila melanogaster*. *Science* 316: 1895–1898

Tomioka, K. & Matsumoto, A. 2010. A comparative view of insect circadian clock systems. *Cell. Mol. Life Sci.* **67**: 1397–406

Tyukmaeva, V.I., Salminen, T.S., Kankare, M., Knott, K.E. & Hoikkala, A. 2011. Adaptation to a seasonally varying environment: a strong latitudinal cline in reproductive diapause combined with high gene flow in *Drosophila montana*. *Ecol. Evol.* 1: 160–168

Vaze, K.M. & Sharma, V.K. 2013. On the adaptive significance of circadian clocks for their owners. *Chronobiol. Int.* 30: 413–433

Wang, X.-P., Yang, Q.-S., Dalin, P., Zhou, X.-M., Luo, Z.-W. & Lei, C.-L. 2011. Geographic variation in photoperiodic diapause induction and diapause intensity in *Sericinus montelus* (Lepidoptera:Papilionidae). *Insect Sci.* 19: 295–302

Weir, B.S. & Cockerham, C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* 38: 1358-1370

Werren, J.H., Richards, S., Desjardins, C. a, Niehuis, O., Gadau, J., Colbourne, J.K., *et al.* 2010. Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. *Science* 327: 343–348

Williams, K.D., Busto, M., Suster, M.L., So, A.K.-C., Ben-Shahar, Y., Leevers, S.J., *et al.* 2006. Natural variation in *Drosophila melanogaster* diapause due to the insulin-regulated PI3-kinase. *Proc. Natl. Acad. Sci. U. S. A.* 103: 15911–5

Winterhalter, W.E. & Mousseau, T. a. 2007. Patterns of phenotypic and genetic variation for the plasticity of diapause incidence. *Evolution* 61: 1520–1531

Yamada, H. & Yamamoto, M.-T. 2011. Association between circadian clock genes and diapause incidence in *Drosophila triauraria*. *PLoS ONE* 6(12): e27493

Yang, D., Lai, X.T., Sun, L. & Xue, F.S. 2007. Parental effects: physiological age, mating pattern, and diapause duration on diapause incidence of progeny in the cabbage beetle, *Colaphellus bowringi* Baly (Coleoptera: Chrysomelidae). *J. Insect Physiol.* 53: 900-908

Yerushalmi, S. & Green, R.M. 2009. Evidence for the adaptive significance of circadian rhythms. *Ecol. Lett.* 12: 970–981

Yerushalmi, S., Yakir, E. & Green, R. 2011. Circadian clocks and adaptation in Arabidopsis. *Mol. Ecol.* 1155–1165

Yuan, Q., Metterville, D., Briscoe, A.D. & Reppert, S.M. 2007. Insect cryptochromes: gene duplication and loss define diverse ways to construct insect circadian clocks. *Mol. Biol. Evol.* 24: 948–955

Summary

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Summary

One of the dominant ecological forces shaping organisms' life is seasonality, the annual change in light, temperature, food availability and other resources necessary for survival, growth and reproduction. How do organisms adapt to seasonal change and fine-tune their life cycles to the environmental cycles? This question constituted the central focus of the research presented in this PhD thesis in which I aimed to deepen knowledge about the genetic basis of seasonal adaptation.

In temperate and polar zones, characterized by strong seasonality, organisms are able to anticipate the approaching seasonal change using specific cues from the environment. One of the most important cues used by many animal and plant species for the forthcoming environmental change is the photoperiod, i.e. the length of the light period in the day. Given its regular progression during the year and its consistency over geological time, photoperiod represents a reliable cue, driving the evolution of a variety of seasonal photoperiodic responses.

Organisms respond to the seasonal change in photoperiod through different physiological and behavioural modifications. For example, many insect species respond to the shortening of the daily light, by entering a physiological state of dormancy, called *diapause,* in which development is arrested and the metabolic activity reduced. Diapause is characterized by an increased resistance to adverse environmental conditions and it is a common strategy used by insects to survive the winter season.

Widely distributed species of plants and animals inhabit a great diversity of seasonal environments on Earth and populations respond

differently to environmental cues so that the organisms' seasonal response matches the specific local seasonal cycle. Hence, geographic variation in seasonality is expected to drive variation in seasonal responses as a result of local adaptation.

In this research, we used natural populations of the jewel wasp *Nasonia vitripennis*, a world-wide distributed species and newly emerging model system for research on adaptation genetics and life history evolution. *Nasonia* is a small parasitic wasp that parasitizes the pupal stage of various fly species. The adult female lays eggs in the host and the eggs develop inside the host puparium into larvae and pupae until they emerge as adults. In this species, the photoperiodic response is a maternally induced larval diapause: when the adult female is exposed to short photoperiods, she lays eggs that develop into diapausing larvae inside the host pupa and only resume development after winter, when conditions are favourable.

In order to study the variation in photoperiodic response in *N. vitripennis*, we collected natural populations from seven locations along a latitudinal gradient in Europe and we studied the diapause response under laboratory controlled conditions, focusing on the maternal sensitive stage (Chapter 2). Under specific light:dark conditions, females produced normal developing offspring during the first days of their life and switched to the production of diapausing offspring after exposure to consecutive photoperiodic cycles. The switch point corresponds to the number of light:dark cycles that need to be experienced by the adult female before diapause is induced in the offspring. In general, the switch point occurred earlier when light cycles were shorter. The individuals from the seven

European populations showed variation in their response and we found a latitudinal cline in switch point for diapause induction: northern females have an early switch point which corresponds to a faster response compared to southern populations. This variation has an adaptive significance: at northern latitudes, characterized by harsh winters and fast changing seasons, individuals are able to respond promptly to the light stimulus to ensure the production of diapause offspring that will survive the winter; on the other hand at southern latitudes the seasonal change is more gradual and the slow response permits the production of non-diapausing offspring which have higher chance to survive and reproduce within the same season.

The clinal variation in switch point suggests that natural selection acts on the maternal sensitive stage and the observed phenotypic variability is based on genetic differences underlying different sensitivities for photoperiodic cues. We suggest that photoperiodic diapause response in *Nasonia* is based on a threshold mechanism in which light:dark cycles are accumulated until the threshold is reached and the diapause is triggered. Differences in the threshold mechanism results in differences in switch point of diapause induction.

In order to elucidate the genetic basis for such variation, we performed reciprocal crosses between individuals from two representative field lines from the most northern and the most southern extremes of our studied cline (Oulu, Finland and Corsica, France) (Chapter 3). Female offspring were screened for their switch point and this was intermediate to the parental lines which showed that variation in photoperiodic induction of diapause has a genetic basis, likely involving multiple loci.

To gain further knowledge about the possible genes and genomic regions involved in diapause response, we used a combination of QTL analysis and candidate gene approach, which allowed the identification of two main genomic regions involved in diapause, located on the first and fifth chromosome of *Nasonia vitripennis*. Interestingly, the highest QTL peak on chromosome 1 corresponds to the candidate clock gene *period*. *Period* is a well-conserved gene from plants, insects, birds to mammals involved in circadian clock mechanisms that regulate various physiological daily activities, entrained by the light:dark cycles.

The involvement of clock genes in seasonal photoperiodic responses has been hypothesized long time ago, but the experiments aiming to test this theory led to contrasting results in various species, leaving the longstanding debate about the possible link between the circadian clock and photoperiodism substantially open. Our finding from the combined QTL analysis and candidate gene approach indicated an involvement of the clock gene *period* in natural variation of photoperiodic diapause, supporting the hypothesis of an evolutionary link between the circadian and seasonal clock. This finding prompted us to further explore the possible role of *period* in photoperiodic diapause induction. In chapter 4 I describe our study on the genetic polymorphism of *period* in *N. vitripennis* natural populations that showed adaptive variation in their photoperiodic response. By sequencing exons of the gene in individuals from the seven clinal European populations, we identified two main alleles, based on non-synonymous SNPs, which showed opposite latitudinal clines in allele frequency. Such clines correlate with the clinal variation in switch point for diapause induction, supporting the hypothesis

of involvement of the *period* locus in adaptive variation of photoperiodic diapause induction.

Since *period* is a clock gene regulating circadian activity, we hypothesized that genetic polymorphism in *period* correlated with variation in diapause response could also correlate to variation in circadian activity in natural populations. We investigated properties of the circadian clock in northern and southern lines, previously used for the genetic analysis and found variation in the level of activity: the northern line was more active than the southern line. Finally, we observed a shallow positive latitudinal cline in the *free running period* (endogenous rhythmicity) in virgin females from the seven studied European populations (Chapter 5).

In the final chapter of my thesis (Chapter 6), I present ideas on the possible mechanistic role of the *period* gene in diapause variation. The two identified *period* alleles might affect the threshold mechanism underlying diapause response in *N. vitripennis*, resulting in observable variation in switch point. Females exposed to specific photoperiodic conditions accumulate a number of light:dark cycles until a threshold is reached and the diapause is induced (switch point). The two *period* alleles might affect either the threshold level (lower for northern populations and higher for southern populations) or the accumulation rate of an unidentified "diapause factor" (faster for northern populations and slower for southern populations) that is needed in a certain amount to trigger the diapause response. In both cases, the phenotypic output is an earlier switch point in northern populations and a later switch point in southern populations. The detailed molecular mechanisms underlying the link between the circadian clock and seasonal photoperiodism, and the specific role of *period*, remain

to be revealed. Future research directed to the characterization of the *Nasonia* clock mechanism is required.

In conclusion, the research presented in this thesis contributes to the general understanding of the evolutionary basis of seasonal adaptation and provides evidence for the involvement of the clock gene *period* in diapause induction, supporting the hypothesis of an evolutionary link between the circadian clock and seasonal photoperiodic response. Future research should focus on the molecular basis of diapause and the circadian clock, and on the identification of other genes involved in the two life history traits.

Samenvatting

Silvia Paolucci[4]

[4] I would like to thank Maartje Giesbers for translating the English summary in Dutch

Samenvatting

Één van de belangrijkste ecologische krachten die het leven van een organisme vorm geeft zijn de seizoenen, de jaarlijkse veranderingen in licht, temperatuur, beschikbaarheid van voedsel en andere middelen die nodig zijn voor overleving, groei en voortplanting. Hoe passen organismen zich aan seizoensverandering aan en hoe stemmen ze hun levenscycli af op veranderingen in de omgeving? Deze vraag staat centraal in het onderzoek dat gepresenteerd wordt in dit PhD proefschrift, waarin ik de kennis over de genetische basis van aanpassingen aan seizoensveranderingen hoop uit te breiden.

In gematigde- en poolgebieden, welke gekarakteriseerd worden door sterke seizoensveranderingen, zijn organismen in staat om te anticiperen op aankomende veranderingen in het seizoen door gebruik te maken van specifieke signalen uit de omgeving. Één van de meest belangrijke signalen waar zowel dieren als planten gebruik van maken om aankomende seizoensveranderingen te voorspellen, is de verandering in fotoperiode, dit is de tijd dat het licht is gedurende de dag. Omdat fotoperiode een regelmaat vertoont gedurende het jaar en consistent is over geologische tijd, geeft het een betrouwbaar signaal dat de drijvende kracht is achter de evolutie van een verscheidenheid aan seizoensgebonden fotoperiodieke reacties.

Organismen reageren door middel van verschillende fysiologische en gedragsmatige veranderingen op de seizoensgebonden veranderingen in fotoperiode. Veel insectensoorten, bijvoorbeeld, reageren op de kortere duur van daglicht door een fysiologische toestand van inactiviteit in te gaan. Dit gedrag wordt diapauze genoemd en houdt in dat de ontwikkeling

tijdelijk wordt stilgezet en de stofwisselingsactiviteit wordt verlaagd. Diapauze wordt gekarakteriseerd door een verhoogde weerstand tegen nadelige omgevingsomstandigheden en is een algemene strategie van veel insecten om de winter te overleven.

Planten en dieren met een breed verspreidingsgebied bewonen een grote verscheidenheid aan “seizoenale” omgevingen op aarde. Plaatselijke populaties reageren hierdoor verschillend op signalen uit de omgeving. Dit heeft als resultaat dat de reactie van een organisme aan de omgevingsverandering precies aansluit op de lokale cyclus in het seizoen. Hierdoor is de verwachting dat geografische variatie in seizoenen de variatie in seizoensgebonden reacties aandrijft als resultaat van lokale adaptatie.

In dit onderzoek maken we gebruik van natuurlijke populaties van de parasitaire wesp *Nasonia vitripennis*. Deze soort komt voor over de hele wereld en is een opkomend modelsysteem voor onderzoek aan genetische adaptaties en de evolutie van “life history” eigenschappen. *Nasonia* is een kleine parasitaire wesp die poppen van verscheidene vliegensoorten parasiteert. Het volwassen *Nasonia* vrouwtje legt eitjes in de gastheer en de eitjes ontwikkelen zich binnen het puparium van de gastheer tot larven en poppen totdat ze uit de gastheer komen als volwassen wespen. In *Nasonia vitripennis* bestaat de fotoperiodieke reactie uit een diapauze van de larven, welke wordt geïnduceerd door de moeder in een receptieve fase: ze legt eitjes die zich ontwikkelen tot larven die in diapauze gaan binnen de gastheer en die pas weer verder ontwikkelen na de winter als de omstandigheden gunstiger zijn.

Om de variatie in fotoperiodieke reactie in *N. vitripennis* te bestuderen,

hebben we natuurlijke populaties verzameld van zeven locaties op verschillende breedtegraden (latitudes) in Europa. We hebben de diapauze reactie bestudeerd onder gecontroleerde omstandigheden in het laboratorium, met als focus de receptieve fase van de moeder. Onder specifieke licht:donker omstandigheden, produceerden vrouwtjes normaal ontwikkelende nakomelingen gedurende de eerste dagen van hun leven. Daarna schakelden de vrouwtjes over op het voortbrengen van diapauze-nakomelingen, nadat ze bloot hadden gestaan aan meerdere opeenvolgende fotoperiodieke cycli. Dit omschakelpunt komt overeen met het aantal licht:donker cycli welke ervaren moet worden door het volwassen vrouwtje voordat diapauze wordt geïnduceerd in de nakomelingen. Over het algemeen vond het omschakelpunt eerder plaats bij vrouwtjes die een kortere licht cyclus ondervonden. De individuen van de zeven Europese populaties vertoonden variatie in hun reactie en we vonden een gradiënt over de breedtegraad, een zogenoemde “latudinale cline”, voor het omschakelpunt van diapauze-inductie: vrouwtjes uit het noorden hebben een vroeg omschakelpunt, wat overeenkomt met een snellere reactie in vergelijking met vrouwtjes uit zuidelijke populaties. Deze variatie heeft een adaptieve betekenis: in noordelijke gebieden, welke gekarakteriseerd worden door strenge winters en snel veranderende seizoenen, zijn individuen in staat om snel te reageren op de lichtstimulus waardoor ze de productie van diapauze nakomelingen garanderen die de winter zullen overleven; aan de andere kant is de seizoensverandering bij gebieden in zuidelijke gebieden geleidelijker en zorgt de langzamere reactie van de vrouwtjes ervoor dat er nakomelingen geboren worden die niet in diapauze gaan en daardoor een kans hebben

om nog in hetzelfde seizoen te overleven en voort te planten.

De clinale variatie in omschakelpunt suggereert dat natuurlijke selectie plaatsvindt tijdens de receptieve fase in de moeder en dat de geobserveerde fenotypische variabiliteit veroorzaakt wordt door genetische verschillen in gevoeligheden voor fotoperiodische signalen. Wij poneren dat fotoperiodische diapauze reactie in *Nasonia* gebaseerd is op een drempel-mechanisme waarin het aantal licht:donker cycli zich ophoopt totdat de drempelwaarde is bereikt en diapauze wordt geïnitieerd. Verschillen in de drempelwaarde resulteren in verschillen in omschakelpunt voor diapauze inductie.

Om de genetische basis van deze variatie op te helderen, hebben we reciproke kruisingen gemaakt tussen individuen van twee representatieve veldlijnen van de meest noordelijke en de meest zuidelijke locatie van de door ons bestudeerde cline (Oulu, Finland en Corsica, Frankrijk) (Hoofdstuk 3). Vrouwelijke nakomelingen werden getest op hun omschakelpunt en deze bleek intermediair aan dat van de ouderlijke lijnen te zijn. Dit toonde aan dat variatie in fotoperiodieke inductie van diapauze een genetische basis heeft, waarbij waarschijnlijk meerdere loci betrokken zijn.

Om meer kennis te verzamelen over de mogelijke genen en genomische locaties die betrokken zijn bij diapauze reactie, hebben we een combinatie gebruikt van een QTL analyse en een kandidaatgen studie. Dit stelde ons in staat om twee grote genetische gebieden te identificeren welke een rol spelen in diapauze en die zijn gelokaliseerd op het eerste en vijfde chromosoom van *Nasonia vitripennis*. Het is interessant om te zien dat de hoogste QTL piek op chromosoom 1 overeenkomt met het

kandidaat klok-gen *period*. *Period* is een geconserveerd gen dat voorkomt bij planten, insecten, vogels en zoogdieren en dat betrokken is bij circadiane klok mechanismen die verscheidene fysiologische dagelijkse activiteiten reguleren, die beïnvloed worden door de licht:donker cycli.

Lang geleden werd al voorgesteld dat klok genen betrokken zouden kunnen zijn bij seizoensgebonden fotoperiodieke reacties, maar experimenten om deze theorie te testen leidden tot elkaar tegensprekende resultaten tussen verschillende geteste soorten. Dit resulteerde in een langdurende discussie over de mogelijke link tussen circadiane klok en fotoperiodiciteit. Onze resultaten, van zowel de QTL analyse als de kandidaatgen aanpak, geven aan dat het klok-gen *period* betrokken is bij natuurlijke variatie in fotoperiodieke diapauze, waarbij het de hypothese ondersteunt dat er een evolutionaire link is tussen de circadiane en seizoenale klok. Deze ontdekking moedigde ons aan om de mogelijke rol van *period* in fotoperiodieke diapauze inductie verder te bestuderen. In hoofdstuk 4 beschrijf ik ons onderzoek aan genetische polymorfie in *period* binnen natuurlijke populaties van *N. vitripennis*, die adaptieve variatie vertoonden in hun fotoperiodieke reactie. Voor individuen van de zeven clinale Europese populaties hebben we exonen van het *period* gen gesequenced. Hiermee konden we twee grote allelen, gebaseerd op niet-synonieme SNPs, identificeren die tegengestelde latudinale clines in allelfrequenties vertoonden. Deze clines correleerden met de clinale variatie in omslagpunt voor diapauze inductie, waarmee we de hypothese ondersteunen dat het *period* locus een rol speelt in adaptive variatie in fotoperiodieke diapauze inductie.

Aangezien *period* een klok gen is dat circadiane activiteit reguleert,

stelden we voor dat genetische polymorfie in *period*, gecorreleerd aan variatie in diapauze reactie, ook gecorreleerd kan zijn aan variatie in circadiane activiteit in natuurlijke populaties. We bestudeerden de eigenschappen van de circadiane klok in de noordelijke en zuidelijke lijnen, die we eerder hadden gebruikt voor de genetische analyse, en vonden variatie in de hoogte van activiteit: de noordelijke lijn was actiever dan de zuidelijke lijn. Tenslotte observeerden we een licht positieve latudinale cline in de *free-running period* (endogene ritmiciteit) in maagdelijke vrouwtjes van de zeven bestudeerde Europese populaties (Hoofdstuk 5).

In het laatste hoofdstuk van mijn proefschrift (Hoofdstuk 6), presenteer ik ideëen over de mogelijke mechanistische rol van het *period* gen in variatie in diapauze. De twee allelen die we geïdentificeerd hebben voor *period* hebben wellicht een invloed op het drempelmechanisme dat diapauze reactie in *N. vitripennis* beïnvloedt, wat kan resulteren in zichtbare variatie in omschakelpunt. Vrouwtjes die bloot staan aan specifieke fotoperiodieke omstandigheden ondervinden een opeenhoping van licht:donker cycli totdat een drempelwaarde bereikt is en diapauze wordt geïnduceerd (omschakelpunt). De twee *period* allelen hebben wellicht invloed op de drempelwaarde (lager voor noordelijke populaties en hoger voor zuidelijke populaties) of op de mate waarin een ongeïdentificeerde “diapauze factor” zich ophoopt (sneller voor noordelijke populaties en langzamer voor zuidelijke populaties) die nodig is om een vastgestelde hoeveelheid te bereiken die de diapauze reactie initieert. In beide gevallen is de fenotypische uitkomst een eerder omschakelpunt in noordelijke populaties en een later omschakelpunt in zuidelijke populaties. De gedetaillerde moleculaire mechanismen die de

link tussen circadiane klok en seizoenale fotoperiodiek beïnvloeden, evenals de specieke rol van het *period* gen moeten nog opgehelderd worden. Hiervoor zal toekomstig onderzoek naar de karakterisatie van het klok mechanisme in *Nasonia* nodig zijn.

Het onderzoek dat ik in dit proefschrift heb gepresenteerd draagt bij aan algemene kennis van de evolutionaire basis van seizoenale aanpassingen en levert bewijs voor de betrokkenheid van het klok-gen *period* in diapauze inductie. Dit ondersteunt de hypothese dat er een evolutionaire link is tussen circadiane klok en seizoenale periodieke reacties. Toekomstig onderzoek zal zich moeten richten op de moleculaire basis van diapauze en circadiane klok, evenals op de identificatie van andere genen die betrokken zijn bij deze twee levensgeschiedenis eigenschappen.

Riassunto

Silvia Paolucci

Riassunto

La stagionalità, ovvero l'alternanza regolare di fattori ambientali legati alle stagioni (quali ad esempio luce, temperatura, e disponibilità di risorse) rappresenta una della maggiori forze selettive che influenzano la vita di piante ed animali sulla terra. Per adattarsi ai cambiamenti stagionali, gli organismi che vivono nelle zone del pianeta caratterizzate da una marcata stagionalità sincronizzano il loro ciclo vitale con il ciclo ambientale, cosí da sfruttare al meglio le risorse disponibili e minimizzare le attività biologiche durante le sragioni fredde. Come fanno tali organismi ad adattarsi al cambiamento stagionale, e come sincronizzano il loro ciclo vitale con il ciclo ambientale? Questa domanda ha rappresentato il punto di partenza del progetto di ricerca del mio Dottorato, durante il quale l'obiettivo principale è stato quello di approfondire la conoscenza dei meccanismi di adattamento ai cicli stagionali e scoprire la base genetica di tale adattamento.

In ambiente caratterizzati dall'alternarsi delle stagioni, gli organismi sono in grado di anticipare il cambiamento stagionale grazie alla percezione di specifici segnali che provengono dall'ambiente. Uno dei più importanti fattori ambientali utilizzati da molte specie di piante e animali come segnale per l'imminente cambiamento ambientale è il fotoperiodo, ovvero la durata del periodo di luce all'interno delle 24 ore di un giorno. Gli organismi rispondono al cambiamento stagionale del fotoperiodo attraverso varie modificazioni fisiologiche e comportamentali. Ad esempio, quando le ore di luce solare in una giornata diminuiscono, molte specie di insetti entrano in una fase fisiologica di quiescenza, chiamata *diapausa*, analoga al letargo dei mammiferi. I cambiamenti fisiologici

dell'insetto durante lo stato di diapausa permettono infatti di sopravvivere alle avverse condizioni ambientali della stagione invernale.

Il mio progetto di ricerca ha avuto come oggetto di studio l'adattamento stagionale di popolazioni naturali di *Nasonia vitripennis,* una specie di vespa parassitoide diffusa in varie aree del pianeta, attualmente utilizzata in numerosi studi di genetica dell'adattamento e biologia evoluzionistica. *Nasonia* é una piccola vespa solitaria, che depone le proprie uova nelle pupe di diverse specie di mosche. Le uova della vespa si sviluppano all'interno della pupa "ospite" ed emergono da essa come vespe adulte alla fine dello sviluppo. La femmina adulta di *Nasonia* è in grado di percepire il cambiamento del fotoperiodo, in modo tale che quando le ore di luce diminuiscono (in corrispondenza dell'inizio della stagione invernale), un segnale (probabilmente di tipo ormonale) viene trasmesso alle uova, che procederanno cosí il loro sviluppo entrando in diapausa durante lo stadio larvale. In questo stato, infatti, avranno una maggiore probabilità di sopravvivere alla stagione invernale.

Per il mio progetto di Dottorato, ho utilizzate popolazioni naturali di vespe campionate in sette località europee lungo un gradiente latitudinale (dalla Finlandia alla Corsica), allo scopo di studiare la variazione nell'adattamento stagionale delle popolazioni originarie di diverse aree del pianeta. Attraverso esperimenti in laboratorio in cui le femmine adulte di *Nasonia* sono state artificialmente esposte a vari fotoperiodi (cicli di luce-notte con un numero predefinito di ore di luce), abbiamo potuto osservate la differenza con cui le femmine delle varie latitudini europee rispondono allo stimolo dato dal fotoperiodo. In particolare, gli individui appartenenti a popolazioni nordiche sono in grado

di indurre la diapausa nella loro progenie in risposta a fotoperiodi più lunghi rispetto a quanto avviene negli individui del sud, ed in modo molto più rapido e netto. Questa variazione nella risposta allo stimolo fotoperiodico è il risultato della selezione naturale che ha portato all'adattamento locale delle popolazioni nel luogo dove vivono. Ad alte latitudini, l'arrivo della stagione avversa è repentino ed avviene durante la fine dell'estate, quando le giornate sono caratterizzate da molte ore di luce. D'altra parte, a basse latitudini il cambiamento stagionale è più graduale ed avviene più tardi nell'anno, quando le giornate (ore di luce) sono relativamente corte. Queste due diverse situazioni ambientali hanno costituito una forte pressione selettiva che ha portato alla differenziazione nella risposta fisiologica (ovvero l'induzione della diapausa) delle popolazioni che vivono a diverse latitudini. Tale pressione selettiva ha modificato infatti la capacità delle femmine adulte di interpretare il segnale ambientale dato dal cambiamento del fotoperiodo, permettendo così una sincronizzazione del ciclo vitale con il ciclo stagionale dell'ambiente.

Il processo evolutivo che porta all'adattamento ad una specifica condizione ambientale per mezzo della selezione naturale è associato a cambiamenti genetici che sono riscontrabili nella sequenza del DNA degli individui adattati. Si parla infatti di “base genetica dell'adattamento”. Per studiare tale base genetica, ho condotto ulteriori esperimenti di laboratorio utilizzando popolazioni di *Nasonia* originarie dei due estremi del gradiente latitudinale considerato: Finlandia e Corsica (Francia). Per prima cosa, ho effettuato degli incroci genetici tra le vespe provenienti da queste due popolazioni sono state incrociate producendo cosí generazioni

“ibride”. Le femmine risultanti da tali incroci sono quindi state esposte a fotoperiodi corti, per determinare la loro capacità di indurre diapausa nella progenie. I risultatati di questi esperimenti hanno confermato che l'abilità di interpretare lo stimolo fotoperiodico ha effettivamente una base genetica ed é dunque risultato della selezione naturale. Per completare lo studio, ho infine individuato l'“impronta genetica” alla base di tale capacità, ovvero il gene coinvolto nella variazione di diapausa. Questo gene, chiamato *period*, fa parte di un gruppo di geni definiti “geni orologio”, responsabili delle attività ritmiche giornaliere (circadiane) controllate dai cicli luce-notte. Diverse varianti nella sequenza del DNA del gene *period* osservate negli individui provenienti dalle popolazioni campionate alle varie latitudini sono infatti associate a diverse risposte di diapausa. Questo risultato indica che *period* ricopre un ruolo importante nell'adattamento stagionale delle popolazioni di *Nasonia* alle varie località, dal momento che è responsabile della diversa interpretazione dello stimolo fotoperiodico. È importante notare come il gene *period* abbia una struttura conservata in vari organismi, dagli insetti ai mammiferi, incluso l'uomo. Tale livello di conservazione su ampia scala evolutiva implica una fondamentale importanza della funzione di questo gene nell'adattamento di molte specie viventi.

Ulteriori ricerche e studi saranno necessari in futuro per capire i dettagli dei meccanismi molecolari alla base della diapausa negli insetti e in generale delle risposte fisiologiche ai cambiamenti stagionali in altre specie animali e vegetali. Attualmente, la ricerca in questo campo è focalizzata sulla caratterizzazione del ruolo di *period* e di altri geni nel processo fisiologico che porta dalla percezione dello stimolo luminoso alla

risposta della diapausa. Inoltre, resta ancora da capire se altri fattori ambientali, come la temperatura, siano utilizzati come segnale del cambiamento stagionale.

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Silvia
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Curriculum vitae

Silvia Paolucci was born in Foligno, Italy, on the 24th of October 1981. In 2006, she received the Master degree in biology (with honours) from the University of Perugia, Italy, with a thesis on the taxonomy and ecology of freshwater bryozoans in a marsh area in Central Italy. After her graduation, she obtained a scholarship from the European funding programme Leonardo da Vinci (project Unipharma Graduates) for an internship at the Cell Biology and Immunology Group of the University of Wageningen, The Netherlands, where she spent 10 months working on fish immunology, and gaining practical experience in molecular biology techniques. In the summer of 2008, she worked in the Animal Ecology Group at the University of Leiden, The Netherlands, taking part in a project on the behavioural biology of cichlid fishes. After this experience, in November 2008 she started her PhD in the Evolutionary Genetics Group at the University of Groningen, The Netherlands, funded by the European programme Marie Curie Initial Training Network SPECIATION. The results of her PhD project are presented in this thesis. Currently, Silvia works as postdoctoral researcher at the Department of Ecology and Evolution of the University of Lausanne, Switzerland, where she studies caste determination in social insects.

List of publications

Hut, R.A., **Paolucci, S.**, Dor, R., Kyriacou, C.P. and Daan,S. 2013. Latitudinal clines: an evolutionary view on biological rhythms*. Proc. R. Soc. B Biol. Sci.* 280: 20130433

Paolucci, S., van de Zande, L. and Beukeboom, L.W. 2013. Adaptive latitudinal cline of photoperiodic diapause induction in the parasitoid *Nasonia vitripennis* in Europe. *J. Evol. Biol.* 26: 705-718

The Marie Curie SPECIATION Network. 2012. What do we need to know about speciation? *Trends in ecology & evolution*. 27: 27–39

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